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Full Length Research Paper

Calcitonin gene-related peptide and substance P significantly influence coronary flow rate in gene knockout mice

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Calcitonin gene-related peptide (CGRP) and substance P (SP) which is potent vasodilator neuropeptides play a counter-regulatory role in several models of experimental hypertension. Langendorff-perfused mouse hearts (n = 328) were used to compare coronary flow rates among wild type (WT), α-CGRP gene knockout (KO) and NK1-KO mice under various pressure loading conditions (20, 30, 40 and 50 mmHg). The aorta of each heart was cannulated and all hearts were perfused with PBS at 37°C. Coronary flow rate was measured by pressure difference of both sides of a capillary. Perfusion was stopped 15 min for ischemia. Deletion of α-CGRP gene resulted in a significant reduction in coronary flow rate for both genders at all pressures. Deletion of NK1 gene resulted in a significant reduction in coronary flow rate for male mice at all pressures, but not for female mice. Coronary flow rate for both WT and α-CGRP-KO mice was consistently lower in female than in male mice, but not for NK1-KO mice. Coronary flow rate in α-CGRP mice was 19.2 and 15.4% lower than that of female and male WT mice, respectively. This effect seems to be gender related with less coronary flow noted in female WT and α-CGRP-KO mice, but not in NK1-KO mice.

Key words: Calcitonin gene-related peptide, flow rate, gene knockout, heart, mouse, neurokinin 1, substance P.

INTRODUCTION

Calcitonin (CT) and calcitonin gene-related peptide (CGRP) are derived from the CT/CGRP gene. CT is a 32 amino acid single chain peptide expressed mainly in the thyroid gland, and CGRP is a 37 amino acid vasoactive neuropeptide that is widely distributed in the central and peripheral nervous systems in mammals (DiPette et al., 1995; Wimalawansa et al., 1996; Ma, 2004; Goadsby, 2008). α-CGRP is produced by the tissue-specific alternative splicing of the primary transcript of the CT/α- CGRP gene and is synthesized almost exclusively in neuronal tissues (Breimer et al., 1988; Rosenfeld, et al., 1983). There is a second CGRP gene, β-CGRP which does not produce CT (Amara et al., 1985).

The two CGRP genes, α and β in rats and I and II in humans, differ in their protein sequences by one and three amino acids respectively, and their biological activities are quite similar in most vascular beds (Breimer et al, 1988).

Substance P (SP) is an 11 amino acids neuropeptide that is abundant in the periphery and the central nervous system, where it is co-localized with other neurotransmitters such as serotonin or dopamine. Also, SP is often co-localized with CGRP in perivascular sensory nerves (DiPette et al., 2000). Three main receptor sub-types for the tachykinins have been identified
(NK1, NK2 and NK3).

Though SP acts primarily as a vasodilator, it also can

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Abbreviations: BP, blood pressure; **CGRP**, calcitonin gene-related peptide; **CT**, calcitonin; **ISP**, immunoreactive substance P; **KO**, gene knockout; **NK1**, neurokinin 1; **NO**, nitric oxide; **PBS**, physiologic buffered solution; **SP**, substance P; **WT**, wild type.

contract smooth muscle. Activation of the SP receptor (also known as the neurokinin 1 [NK1] receptor) causes oxidative stress and neurogenic inflammation (Weglicki et al., 1996). As a result, studies typically involve receptor knockouts as opposed to SP gene knockouts to eliminate any possible complication of oxidative stress. As a member of the tachykinin family, SP is derived from tissue-specific alternative splicing of the preprotachykinin I gene and is produced almost exclusively in neuronal tissues (Hokfelt et al., 2000). A prominent site for SP synthesis is the dorsal root ganglia which contain the cell bodies of sensory neurons that terminate centrally in the spinal cord and peripherally on blood vessels (DiPette, et al., 2000). SP and its receptor are widely distributed in the central and peripheral nervous system. Immunoreactive substance P (ISP) containing nerve fibers is widely distributed in the brain as well as peripheral sensory nerve endings, where it is often co-localized and co-released with CGRP (Mau et al., 1999). SP is involved in various physiological activities such as neuromodulation, smooth muscle contraction and the modulation of vascular tone (Stewart-Lee et al., 1989; Whittle et al., 1989; Khurshed et al., 2002). SP acts only in situations with an increased nervous activity, for example, after stress-induced activation of the pituitary-adrenal and sympathetic-adrenal axis. It normalizes stress-induced disorders by maintaining homeostasis in the catecholamine system. The basis of the antistress effect is the modulation of both biosynthesis and release of catecholamines in the adrenals. The homeostasis effect of SP, as well as the antistress effect does not require the complete sequence of the molecule. The N-terminal SP fragment is fully active and has no side effects (Oehme et al., 1987).

SP is a nitric oxide (NO)-dependent vasodilator that influences the relationship of mesenteric arterial arcades flow rate and NO release (Keber et al., 2001). It was reported that CGRP and SP play an important role in the development of pain and hyperalgesia (Greco et al., 2008). Statins act directly on sensory neurons to decrease expression of proinflammatory neuropeptides that trigger neurogenic inflammation, specifically CGRP and SP (Bucelli et al., 2008).

In this study, Langendorff-perfused heart preparations under both non-ischemia and ischemia conditions were used to compare coronary flow rates among wild type (WT) control, α-CGRP gene knockout (α-CGRP-KO) and SP gene knockout (SP-KO) mice with different genders under various pressure loading conditions (20, 30, 40 and 50 mmHg) to determine the effects of α-CGRP and SP on coronary flow rate.

MATERIALS AND METHODS

Animals

Three hundred and twenty-eight (328) mice were used in the study (2 - 3 months, 23.4 ± 2.5 g). Homozygous α-CGRP-KO and NK1-KO mice (C57BL/6, Fayetteville, NC, USA) were obtained from breeding pairs generated by the genetic targeting of the α-CGRP and NK1 genes respectively. These gene knockout mice were provided by Dr. Robert Gagel at the Baylor College of Medicine (Hoff et al., 1998).

Heart weight

To obtain an accurate heart weight is important, but to weigh the heart after adsorption of water usually damages the heart and calculation of the heart weight from body weight can cause substantial error. Instead, we used the watery heart weight to calculate the real heart weight (net weight). The hearts from 60 mice were removed after mice were sacrificed and put in ice cooled physiologic buffered solution (PBS) and the watery hearts from the buffer were weighed immediately. The hearts were adsorbed with paper to obtain the net heart weight.. Ratio of the net heart weight to watery heart weight was obtained from the 60 mice. The watery heart was weighed for each experimental heart and the real heart weight was calculated by the calibration of the 60 mice.

Heart perfusion

Fifty-two mouse hearts were used in the perfusion to measure flow rate (9 female WT, 6 female α-CGRP-KO, 5 female NK1-KO, 10 male WT, 8 male α-CGRP-KO, and 14 male NK1-KO). Mice were injected with heparin (Sigma Chemical Co., St. Louis, MO, USA) at 1000 units/kg (I.P.). Thirty min after heparin injection, mice were anesthetized with pentobarbital at 100 mg/kg mouse (Abbott Laboratories, North Chicago, IL, USA). Hearts were immediately isolated and placed in oxygenated ice cooled PBS. Then the aortas of the isolated hearts were cannulated and hearts placed in an incubator with oxygenized PBS at 37°C. The hearts were perfused with oxygenized PBS by the Langendorff method. The PBS perfusion was performed from aorta to coronary, left atrium and flow out through the left pulmonary veins. The Langendorff-perfused heart preparations were used to compare coronary flow rates among WT, α-CGRP-KO and NK1-KO mice for both genders under various pressure loading conditions (20, 30, 40 and 50 mmHg) (Figure 1).

Flow rate

One hundred and twenty-seven mice were used in the coronary flow rate measurements (19 female WT, 25 female α-CGRP-KO, 17 female NK1-KO, 27 male WT, 20 male α-CGRP-KO and 19 male NK1-KO). Coronary flow rate was measured by the pressure difference of both sides of a capillary in the perfusion line. Flow rate vs. pressure difference was calculated using PBS. The relationship of coronary flow rate and pressure was measured at multiple perfusion pressures and the relationship curve was constructed. The flow rates of WT, α-CGRP-KO and NK1-KO mouse hearts for both genders were compared.

Ischemia

One hundred and forty-one mice were used in the ischemia measurements (18 female WT, 19 female α-CGRP-KO, 17 female NK1-KO, 25 male WT, 38 male α-CGRP-KO, and 24 male NK1-KO). Hearts were exposed to ischemia situation by stopping perfusion for 15 min after the hearts were fixed and perfused one hour with oxygenized PBS by the Langendorff method. As heparin changes the heart behavior in ischemia, heparin was not injected for this test and the blood inside the heart was washed out quickly with PBS after the heart was removed from the mouse. Then the

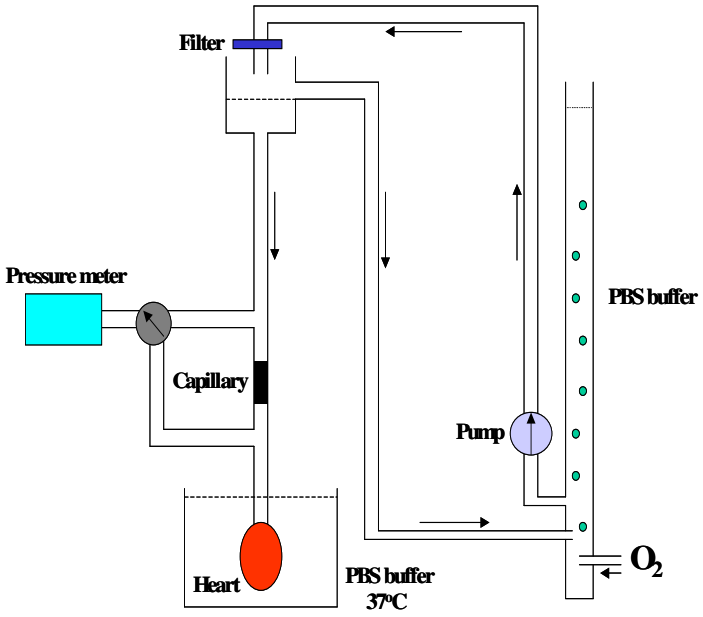

Figure 1. Langendorff Heart Preparation Set Up for Coronary Flow Rate Measurement.

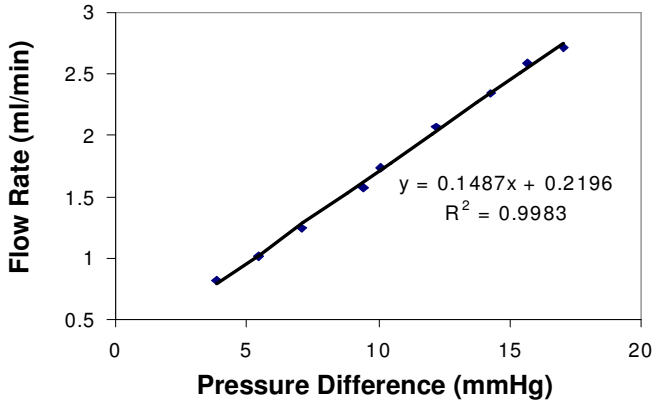

Figure 2. Correlation between Mouse Wet Weight and Net Weight.

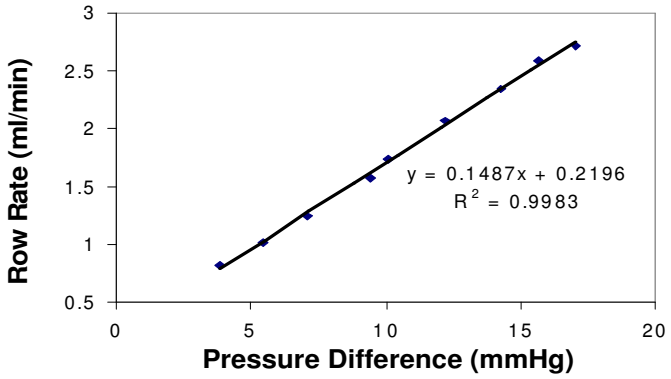

Figure 3. Flow rate vs. Pressure Difference Calculated with PBS.

hearts were perfused with oxygenized PBS. The flow rates of WT and CGRP-KO mice for both genders and SP-KO male mouse hearts were compared.

RESULTS

Heart weight: The average watery and net mouse heart weights obtained from 60 mice were 0.158 ± 0.019 and 0.131 ± 0.016 respectively, and the ratio of the net heart weight to watery heart weight was 0.83. There was a linear relationship of the watery and net heart weight ($y = 0.8316x$; $R^2 = 0.793$) (Figure 2).

Flow rate calibration

There was a linear relationship between the flow rate calibrated by PBS and the pressure difference of both sides of a capillary in the perfusion line ($y = 0.1487x + 0.2196$; $R^2 = 0.9983$) (Figure 3).

Flow rate

As shown in Tables 1 and 2, knockout of the α-CGRP gene resulted in a significant reduction in the coronary flow rate for both genders at all pressures (average of 6.13 ± 1.24 from 7.59 ± 0.95 ml/min/g net heart weight for female, $p < 0.0005$ and 6.80 ± 0.77 from 8.04 ± 1.27 ml/min/g net heart weight for male, $p < 0.01$). Deletion of the NK1 gene resulted in a significant reduction in the coronary flow rate for male mice at all pressures (average of 7.34 ± 1.05 from 8.04 ± 1.27 ml/min/g net heart weight, $p < 0.0008$), but not for female mice. The female mouse coronary flow rate for NK1 increased to 9.18 ± 1.40 ml/min/g net heart weight compared to 7.59 ± 0.95 ml/min/g net heart weight of WT. In addition, the coronary flow rate for both WT and α-CGRP-KO mice was consistently lower in female than in male mice (average of 7.59±0.95 to 8.04 ± 1.27 ml/min/g net heart weight for WT and 6.13 ± 1.24 to 6.80 ± 0.77 ml/min/g net heart weight for α-CGRP-KO, and was significant at higher perfusion pressure), but not for SP-KO mice which had a higher flow rate (average of 9.18 ± 1.40 to 7.34 ± 1.05 ml/min/g net heart weight for NK1, $p < 0.0002$). The data show that the coronary flow rate for α-CGRP mice is 19.2% lower than that of WT mice for females and 15.4% for males. Also, this effect seems to be gender related with less coronary flow noted in the female mice for WT and α-CGRP-KO, but not for SP-KO (Tables 1 and 2).

Ischemia

In this experiment, we measured the ratios of mouse coronary flow rates after and before ischemia to evaluate the heart reaction to ischemia. The ischemia was done by 15 min stopping perfusion. The results showed that

Table 1. Mouse Coronary Flow Rate (ml/min/g net heart weight) vs. Perfusion Pressure.

Perfusion Pressure (mmHg)	WT Female n=25	α-CGRP-KO Female N=19	NK1-KO Female n=17	WT Male n=20	α-CGRP-KO Male n=27	NK1-KO Male n=19
50	11.42±1.42	9.76±1.80	13.75±1.78	12.36±1.75	10.54±0.94	11.51±1.54
40	9.15±1.06	7.73±1.45	11.00±1.57	9.85±1.34	8.23±0.78	8.84±1.22
30	6.31±0.79	4.88±0.92	7.49±1.22	6.52±1.22	5.53±0.75	5.95±0.88
20	3.48±0.53	2.16±0.82	4.48±1.02	3.43±0.78	2.92±0.61	3.08±0.26
Mean	7.59±0.95	6.13±1.24	9.18±1.40	8.04±1.27	6.80±0.77	7.34±1.05

Table 2. P Value between the Different Mouse Coronary Flow Rates.

Perfusion Pressure (mmHg)	F WT /F CGRP-KO	F WT/F NK1-KO	F CGRP-KO/F NK1-KO	M WT /M CGRP-KO	M WT/M NK1-KO	M CGRP-KO /M NK1-KO	F WT/M WT	FCGRP-KO/M CGRP-KO	F NK1-KO/M NK1-KO
50	0.0009	0.00005	0.00000006	0.0001	0.0573	0.0106	0.0312	0.0371	0.0002
40	0.0005	0.0001	0.0000001	0.00003	0.0095	0.0336	0.0335	0.0734	0.00004
30	0.000003	0.0008	0.00000003	0.0016	0.0502	0.0503	0.2503	0.0071	0.00009
20	0.0004	0.0006	0.0000009	0.0098	0.0583	0.1708	0.4173	0.1126	0.00002

Note: F – female; M – male

female α-CGRP-KO mice had a lower ratio than WT mice and that NK1-KO mice had a higher ratio. For males, both α-CGRP-KO and NK1-KO mice had lower ratios than WT mice, and α-CGRP-KO mice showed the lowest (Figure 4, Table 3).

DISCUSSION

CGRP and SP are two vasoactive neuropeptides that play counter regulatory roles in hypertension and coronary flow rate. CGRP causes peripheral arterial dilation, thereby decreasing blood pressure and increasing heart rate in a dose-dependent manner (DiPette, et al, 1995; Wimalawansa, et al, 1996). CGRP is a peptide that influences the blood pressure. CGRP has several important physiologic roles: (1) it is a potent vasodilator and can affect the force and rate of heart beat. (2) it can modulate acetylcholine receptor function at the neuromuscular junction. (3) It has been demonstrated to block tolerance to morphine and (4) it can modulate antigen presentation in Langerhans cells in the skin. Despite these important physiological functions, therapeutic strategies using CGRP have been impeded due to the lack of a cloned CGRP receptor with which ligands could be developed. There is an induction of the blood pressure when animals are lacking CGRP. The CGRP neuropeptide has a marked dose-dependent decrease in blood pressure with increases in heart rate and blood pressure of CGRP gene knockout mice (Gangula et al., 2000). CT gene was first cloned in 1980 (Jacobs et al., 1981) and CGRP was discovered in 1982 by molecular cloning of CT gene (Amara et al., 1982; Rosenfeld et al., 1983). The CT mRNA predominates in the thyroid while the CGRP-specific mRNA appears to predominate in the hypothalamus (brain). The second rat CGRP (β-rCGRP) gene has been discovered in brain and thyroid. This β-rCGRP is different from the α-CGRP by one amino acid (position 1 Ala instead of Ser). The second human CGRP (β-hCGRP) gene has been discovered in medullary thyroid (Tschopp et al., 1985). This β-hCGRP differs from the α-hCGRP by three amino acids located position 3, 22 and 25. Different splicing of primary RNA transcript arouses the translation of CT and CGRP peptides in a tissue-specific manner (Ma, 2004).

According to Okamoto et al. (2003) ketamine and pentobarbital inhibit SP receptor function, and the mechanism of their inhibition on SP receptor function could not be through activation of the protein kinase C pathway and may be due to noncompetitive displacing of SP binding (Okamoto et al., 2003).

α-CGRP plays a major role in coronary blood flow. From the research results here, we showed that flow rates of both α-CGRP-KO and SP-KO mouse hearts are lower than those of WT mouse hearts. In the ischemia condition, the flow rate ratios of both α-CGRP-KO and SP-KO mouse hearts are lower than those of WT mouse hearts for males, but there is no difference between α-CGRP-KO and WT mouse hearts for females. This indicates a difference in females and males when α-CGRP-KO is genetically deleted. The results of this study

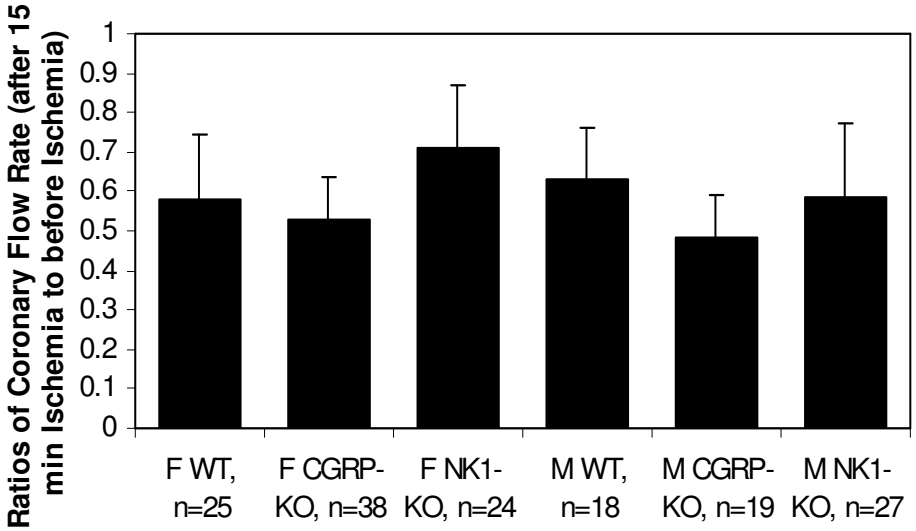

Figure 4. Male Mouse Coronary Flow Rate and Beating after Ischemia.

Table 3. P Value between the Ratio of Mouse Coronary Flow Rates after and before Ischemia.

F WT/F CGRP-KO	F WT/F NK1-KO	F CGRP-KO/F NK1-KO	M WT/M CGRP-KO	M WT/M NK1-KO	M CGRP-KO /M NK1-KO	F WT/M WT	F CGRP-KO/M CGRP-KO	F NK1-KO/M NK1-KO
0.13	0.01	0.0002	0.00005	0.15	0.01	0.01	0.091	0.01

Note: F – female; M – male.

provide information that α-CGRP and SP or their activetors/enhancers may be candidates to improve blood flow rate. These potential pharmaceutical applications could be considered in clinical trials on ischemia or hypertension, etc. The gender correlation of CGRP and CT on flow rate offers information that it may be necessary to treat female and male patients differently. Lastly, it is possible to consider clinical treatment by a combination of CGRP and SP.

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