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Proinsulin C-peptide activates vagus efferent output in rats.

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Footnotes

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Abbreviations: α -MT, DL- α -methyl-*p*-tyrosine methyl ester; BAT, brown adipose tissue; DHBA, dihydroxybenzylamine; NE, norepinephrine; NO, nitric oxide.

Abstract

The aim of this study was to examine the effect of proinsulin C-peptide on the autonomic nervous systems in rats. Intravenous administration of C-peptide gradually increased electrophysiological activity of the vagus nerves into the stomach and pancreas for at least 90 min. It also slightly increased gastric acid secretion that was suppressed by the treatment with atropine. Intraperitoneal injection of C-peptide did not affect the basal and stress-induced norepinephrine turnover rate, a biochemical index of sympathetic nerve activity. These results indicate that C-peptide increases parasympathetic nerve activity without affecting sympathetic nerve activity. This could explain, at least in part, the ameliorating effects of C-peptide on impaired cardiac autonomic nerve functions in patients with type 1 diabetes.

Keywords: atropine, C-peptide, gastric acid, norepinephrine, parasympathetic nerve, sympathetic nerve, vagus.

1. Introduction

Diabetic neuropathy is an important feature of patients with diabetes, and is defined by the presence of detectable sensory, motor, and autonomic deficits on clinical examination, irrespective of the presence or absence of overt symptoms [23]. Among others, dysfunction of the autonomic nervous system is associated with increased risk of mortality [34]. Cardiac sympathetic dysfunction is commonly present in not only diabetic patients but also animal models with altered nerve profiles and reduced norepinephrine (NE) turnover and functions [25, 33, 34]. Similarly, cardiac autonomic neuropathy with reduced vagal tone occurs early and is frequently found in diabetic patients [16, 26, 27, 32].

C-peptide, a connecting segment of proinsulin, is secreted from pancreatic β-cells into the circulation along with insulin. Plasma C-peptide levels are often used as a marker of actual insulin secretion, and inversely related to the development of diabetic complications including neuropathy [22, 28, 29]. For a long time, C-peptide had been considered to be biological inert. However, recent studies have shown that C-peptide is an active peptide hormone with potentially important physiological effects against diabetic complications in patients with type 1 diabetes and experimental models [2, 4, 8, 10, 11, 22, 28, 29]. For example, short-term infusion of C-peptide improves impaired cardiac autonomic nerve functions, mainly parasympathetic components, in patients with polyneuropathy and type 1 diabetes [10]. Long-term (3 month) administration of C-peptide to patients with early sign of diabetic neuropathy ameliorates sensory nerve dysfunction [4]. However, the mechanism behind the beneficial effects of C-peptide on diabetic neuropathy is not fully understood.

Recently, a nitric oxide (NO)-sensitive vascular response has been proposed to

correct impaired nerve nutritive perfusion and sensory nerve conduction velocity [2]. Indeed, C-peptide acts on endothelial cells to stimulate Ca^{2+}-dependent NO synthase activity [30] and also its transcription through the mitogen-activated protein kinase pathways [13, 14]. In addition to these NO-dependent C-peptide action, our previous results that antagonistic effect of C-peptide on sympathetically mediated response was blocked by prior treatment with atropine, a parasympatholytic drug, [20] suggest that C-peptide might directly activate the parasympathetic nervous system and also influence indirectly the sympathetic nervous system or its effector function.

To test this hypothesis, we here examined the effects of C-peptide on electrophysiological vagal activity, gastric acid secretion that can be increased by parasympathetic activity [18], and NE turnover rate as a biochemical index of sympathetic nerve activity [1, 21, 24, 33].

2. Materials and methods

2.1. Materials

Human C-peptide was generously provided by Eli Lilly Co. (Indianapolis, IN, USA) and dissolved in sterilized phosphate buffered saline (PBS). Atropine sulfate was obtained from Tanabe Pharmaceutical Co. (Osaka, Japan). DL- α -methyl-*p*-tyrosine methyl ester (α -MT) and dihydroxybenzylamine (DHBA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other reagents were of analytical grade.

2.2. Experimental animals

Rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). Female Wistar rats weighing 170-220 g were used for NE turnover experiments, in line with previous experiments [20]. Male Wistar rats weighing 280-320 g were used for electrophysiological study and measurement of gastric acid secretion. They were housed at 24 °C with a 12:12 h light: dark cycle (light on 07:00-19:00) and given laboratory chow and water *ad lib*. The experimental procedure and care of animals were in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University.

2.3. NE turnover

Norepinephrine (NE) turnover was assessed from a decrease in tissue NE concentration after the inhibition of catecholamine biosynthesis with α -MT, essentially as described previously [1, 21, 24]. In an experiment for cold stress, rats were injected intraperitoneally with either α -MT (300mg/kg) and saline (n=12) or α -MT and C-peptide (1 mg/kg) (n=12), and half of the rats with each treatment (n=6 per group) were immediately transferred to a cold room at 4°C. Six hour after the α -MT injection, the animals were decapitated and the spleen, lung, heart and interscapular brown adipose tissue (BAT) were removed and stored at –80°C until assay of NE content. Another six

rats were sacrificed immediately after injection of α -MT alone to measure initial NE content at time 0. In an experiment for footshock stress, rats were injected as described above and half of the rats with each treatment (n=6 per group) were given a single 6-h session during which electrical footshocks were applied for 2 sec every 1 min as previously described [20]. The tissues were then collected for NE assay.

For NE assay, tissue was homogenized in ice-cold 0.2M perchloric acid solution containing DHBA as an internal standard, and centrifuged to remove acid insoluble materials. After adjusting pH of the supernatant to 8.5 with Tris base, activated alumina (Nacalai Tesque, Kyoto, Japan) was added to the supernatant, mixed gently for 10min, and washed three times with distilled water. NE and DHBA bound to alumina were then eluted with 0.2M acetic acid, and analyzed using HPLC system with an electrochemical detector (Eicom, Kyoto, Japan). Fractional turnover rate (κ) of NE was calculated by a regression line of the *ln* NE content versus time. NE turnover rate was estimated as the product of the κ times the initial NE content.

2.4. Electrophysiological study on vagal nerve activity

Rats were anesthetized by intraperitoneal injection of urethane (1g/Kg) and kept warm with a heating pad. The efferent nerve activities were recorded from a nerve filament dissected from the central cut end of the vagus nerve innervating the stomach and the pancreas as described [17]. The recording electrodes were immersed in a mixture of liquid paraffin and petroleum jelly to prevent dehydration. The nerve activity was recorded on magnetic tape, and analyzed by using a window discriminator, which separated discharges from background noise, and a rate meter for counting number of impulse in 5sec. The mean discharge rates of 10 successive measured samples were compared before and after the intravenous injection of C-peptide (1~100ng/ animal).

2.5. Gastric acid secretion

Rats were deprived of food for 24 - 36 h prior to the experiments, and anesthetized by urethane injection (1 g/Kg) and kept warm with a heating pad. Gastric acid secretion was determined according to Masuda et al. [15] with minor modifications. Briefly, a trachea was cannulated and the esophagus was ligated by silk thread. Abdomen was incised and the stomach and duodenum were exposed. The gastric lumen was then perfused with saline adjusted to pH 7.0 at 37°C at a flow rate of 1.0 ml/min, from an acute gastric fistula made in the forestomach to the cannula inserted through the pylorus. The perfusate from the stomach was collected every 5min and titrated to pH 7.0 using a pH meter with 10 mM NaOH. C-peptide (10μg/animal) was injected from jugular vein and atropine (0.25mg/animal) was given into an abdominal space.

2.6. Data analysis

All data were expressed as means ± SEM. Statistical comparison were made by ANOVA with a post hoc test using the Newman-Keuls multiple range test or Student's *t*-test.

3. Results

To investigate the possible direct effect of C-peptide on the sympathetic nerve activity, the tissue NE turnover in some peripheral organs was assessed after injection of C-peptide in rats subjected to footshock stress and cold exposure. As shown in Table 1, the tissue NE content at time 0 varies considerably among organs, roughly reflecting the density of sympathetic innervation of each organ. In the interscapular BAT, NE content decreased after inhibition of tyrosine hydroxylase by α -MT by 40% in controls (623.4 ± 14.7 versus 371.3 ± 40.3 ng/g of organ, $p<0.05$), and lowered further during exposure to cold (194.1 ± 8.5 ng/g of organ, $p<0.05$ compared with the control group). The turnover rate of NE estimated from the decrement of NE content was 42 and 71.6 ng/g/h in the control and cold-exposed groups, respectively. Thus, exposure of rat to cold accelerates NE turnover in the BAT, the major site for sympathetically regulated thermogenesis during the cold stress. In contrast, NE turnover in the spleen was not affected by the cold exposure, while those in lung and heart were accelerated similarly to BAT (Table 1). Neither NE turnover in all organs tested nor its acceleration by cold exposure was affected by C-peptide injection (Table 1).

When given footshock stress, NE content in the spleen decreased much more than that of the control (251.4 ± 11.3 versus 88.2 ± 16.3 ng/g of organ, $p<0.05$). The NE turnover rate was 26.9 and 54.1 ng/g/h in the control and the footshock stress groups, respectively (Table 2). Similar increases in NE turnover were found in the lung and heart. However, different from the results obtained by the cold stress, NE turnover in BAT was not influenced by the footshock stress (Table 2). C-peptide injection did not affect NE turnover in both the control and footshock stress groups (Table 2).

To test whether C-peptide affects the vagal activities, multi-unit discharges were recorded from nerve filaments isolated from the central cut end of the vagal branches

innervating the stomach and pancreas. As shown in Fig. 1A, the rate of spontaneous discharge recorded from the vagal branch to the pancreas tended to increase 60min after intravenous injection of C-peptide (10 ng) and was enhanced significantly by additional injections of C-peptide (100 ng). Moreover, the spontaneous discharge rate from the vagal branch to the stomach also tended to increase 30min after administration of C-peptide and agumented significantly at 90 min (Fig. 1B). No significant change in the discharge rate was produced by injection of physiological saline (not shown).

We next examined the effects of C-peptide on gastric acid secretion. As shown in Fig. 2A, gastric acid secretion was evoked immediately after intravenous injection of gastrin but not of saline, and peaked at 20 min and lasted to 60 min. When given C-peptide, no detectable increase of gastric acid secretion was seen until 60min after the injection, while slight but significant increases were observed thereafter ($p<0.05$) (Fig.2B and 2C). Administration of atropine did not affect gastric acid secretion itself, but inhibited the C-peptide-induced increase (Fig.2C).

4. Discussion

It was reported that the application of footshock stress to rats produced a marked suppression of mitogenic response of splenic lymphocytes, which was attenuated by either surgical denervation of the splenic nerve or β-adrenergic blockade [3, 19, 31]. These findings suggested that NE released from sympathetic nerve terminals acts through the β-adrenergic receptors on the surface of splenic lymphocytes to lower their cell proliferation. Since administration of C-peptide obviated the footshock stress-induced suppression of the mitogenic response [20], it was assumed that C-peptide might alter sympathetic nerve activity. However, the present findings that C-peptide did not affect the basal and the footshock stress-induced tissue-specific acceleration of NE turnover suggest that C-peptide does not influence the sympathetic nerve system itself and that the antagonistic effect of C-peptide is on the steps distal to NE release.

The antagonistic effect of C-peptide on the sympathetically mediated response disappeared when atropine, a parasympatholytic agent, was given [20]. Thus, it is possible that C-peptide might be an activator of the parasympathetic nervous system. Indeed, in the present study, C-peptide was shown to increase the rate of discharge of the vagus nerves innervating the stomach and pancreas. Moreover, it significantly increased gastric acid output, which was under the control of vagal nerve [18] and inhibited by atropine. These results are in line with a report of patients with diabetic autonomic neuropathy, where C-peptide improves the reduced heart rate variability during deep breathing, an indicator of primarily vagal nerve activity [10]. Therefore, it is suggested that C-peptide is a parasympathetic stimulant not only in rodents but also in human.

It is well documented that both vagal and sympathetic nerves innervate heart and alter cardiac functions. That is, sympathetic activation increases heart rate

(chronotropy), inotropy and conduction velocity (dromotropy), whereas parasympathetic stimulation shows opposite effects. Although C-peptide failed to suppress NE release in the heart as well as the spleen, it is likely that vagal activation by C-peptide reduces the sympathetic tone on the cardiac functions. This may explain some of the C-peptide actions in patients with type 1diabetes such as improvement of heart rate variability [10] and increased blood flow of myocardium [7, 12].

It is known that parasympathetic nerves innervate blood vessels in some specified tissues including gastrointestinal tract, where the vagus nerve causes vasodilation through the atropine-sensitive muscarinic receptor system coupled to NO formation. However, it should be noted that exogenously administrated C peptide is reported to have no effect on tissue blood flow in healthy humans, and that parasympathetic vasodilatory fibres are not known to exist in humans. Therefore, C-peptide-induced increase of blood flow in kidney and skin observed in patients with type 1 diabetes [5, 9] may be attributed to vasodilatory effect of C-peptide through directly acting on vascular endothelial cells [13, 14, 30].

The mechanism(s) by which C-peptide activates the parasympathetic nervous system remains to be elucidated. However, the antagonistic effect of C-peptide on the sympathetically mediated splenic response was achieved not only by peripheral administration but also by injection of limited amounts into the cerebroventricule [20], suggesting that C-peptide present in the brain [6] or derived from blood circulation may exert its function directly in the central nervous system.

The mechanism(s) through which C-peptide obviates on the sympathetically mediated response through the parasympathetic nervous system in rats [20] is not fully understood. As C-peptide most probably increases in visceral blood flow through the parasympathetic nervous system in rodents and/or its direct vasodilatory action, one

possible mechanism is that increased blood flow in the spleen lowers concentrations of NE sufficient to cause the response.

In conclusion, the data demonstrated that C-peptide activates parasympathetic nerve activity without influencing NE release from sympathetic nerve terminals. As discussed above, parasympathetic activation may explain, at least a part, the beneficial effects of C-peptide on patients with type 1 diabetes, in addition to enhancement of local NO production.

Acknowledgement

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Table 1. Effect of C-peptide on tissue norepinephrine turnover in various organs of rats exposed to cold.

Time	0	6h			
Treatment		Saline		C-peptide	
Cold exposure		-	+	-	+
Spleen					
NE	343.3±11.7	229.6±25.9	270.0±15.2	270.0±15.2	230.4±18.5
κ		7.3±2.1	6.4±1.4	4.1±1.1	5.8±1.2
BAT					
NE	623.4±14.7	371.3±40.3	194.1±8.5*	366.9±6.0	198.2±26.5*
κ		9.1±1.8	19.5±0.8*	8.8±0.5	19.6±1.9*
Heart					
NE	352.4±15.9	240.0±13.3	117.9±15.2*	232.7±9.9	118.2±13.5*
κ		5.5±1.3	17.9±2.3*	5.9±1.1	17.8±2.3*
Lung					
NE	62.7±6.2	33.5±5.1	21.1±1.6*	31.7±2.2	18.8±2.8*
κ		8.3±2.7	19.0±2.0*	10.2±2.1	20.6±2.9*

Rats were injected α-MT (300mg/kg) with saline or C-peptide (1mg/Kg) intraperitoneally, and kept at either 22°C (-) or 4°C (+) for 6h. The tissue was then excised and NE contents (ng/g organ) were determined. The κ is a fractional turnover rate obtained by changes in NE content. Values are means ± SEM for 6 rats. *p<0.05, compared with the saline control without C-peptide injection and cold exposure.

Table 2. Effect of C-peptide on tissue norepinephrine turnover in various organs of rats given footshock stress.

Time	**0**	**6h**			
Treatment		**Saline**		**C-peptide**	
Cold exposure		**-**	**+**	**-**	**+**
Spleen					
NE	412.8±14.9	251.4±11.3	88.2±16.3*	256.7±11.7	90.8±18.9*
κ		8.3±1.0	27.5±3.9 *	8.0±1.0	27.5±4.3 *
BAT					
NE	583.7±28.1	244.2±36.3	235.7±11.5	230.4±31.3	206.5±19.8
κ		15.3±2.5	15.1±1.2	16.0±2.1	17.7±2.0
Heart					
NE	360.1±7.1	198.7±7.7	91.3±10.6*	215.7±9.5	85.2±9.3*
κ		10.0±0.7	23.5±2.1 *	8.6±0.8	24.6±2.2*
Lung					
NE	81.5±2.9	46.1±2.8	27.2±2.2*	43.9±3.3	27.3±1.4*
κ		9.6±1.2	18.5±1.5*	10.5±1.5	18.2±1.0*

Rats were injected α-MT (300mg/kg) with saline or C-peptide (1mg/Kg) intraperitoneally, and given footshock stress (+) for 6h or no stress treatment (-). Subsequently, tissue NE contents (ng/g organ) were determined. The κ is a fractional turnover rate obtained by changes in NE content Values are means ± SEM for 6 rats. *p<0.05, compared with the saline control without C-peptide injection and footshock stress.

Figure legends

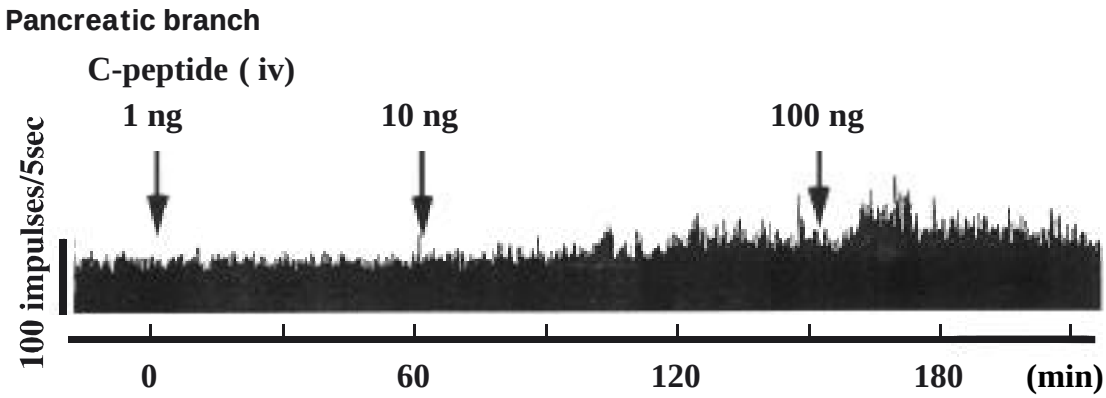
Fig. 1. Effects of C-peptide on vagal nerve activity in rats.

The efferent nerve activities were recorded from a nerve filament dissected from the central cut end of the vagus nerve innervating into the pancreas (A) and stomach (B). Typical examples of recording and the average of the mean discharge rates from 5 rats were compared before and after the intravenous injection of C-peptide (1~100 ng/animal). Values are means ± SEM. *$p<0.05$, compared with the value before C-peptide injection.

Fig. 2. Effects of C-peptide on gastric acid secretion in rats.

(A, B) Gastrin (1μg/animal, open square in A), saline (open circle in A and B) and C-peptide (10μg/animal, closed circle in B) were injected from jugular vein and the time of the injection as indicated by arrow was referred to Zero. Gastric acid output in every 5min was determined as described in Materials and methods and values are means ± SEM for 3~8 rats. (C) C-peptide (closed symbols) and saline (open symbols) were injected with or without atropine (0.25mg/animal, squares and circles, respectively) as in B, and shown are changes in gastric acid output from that produced 60 min after the injection. *$p<0.05$, compared with saline control.

Fig. 1A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10 ng
100 ng
100 impulses/5sec
0
60
120
180
(min)

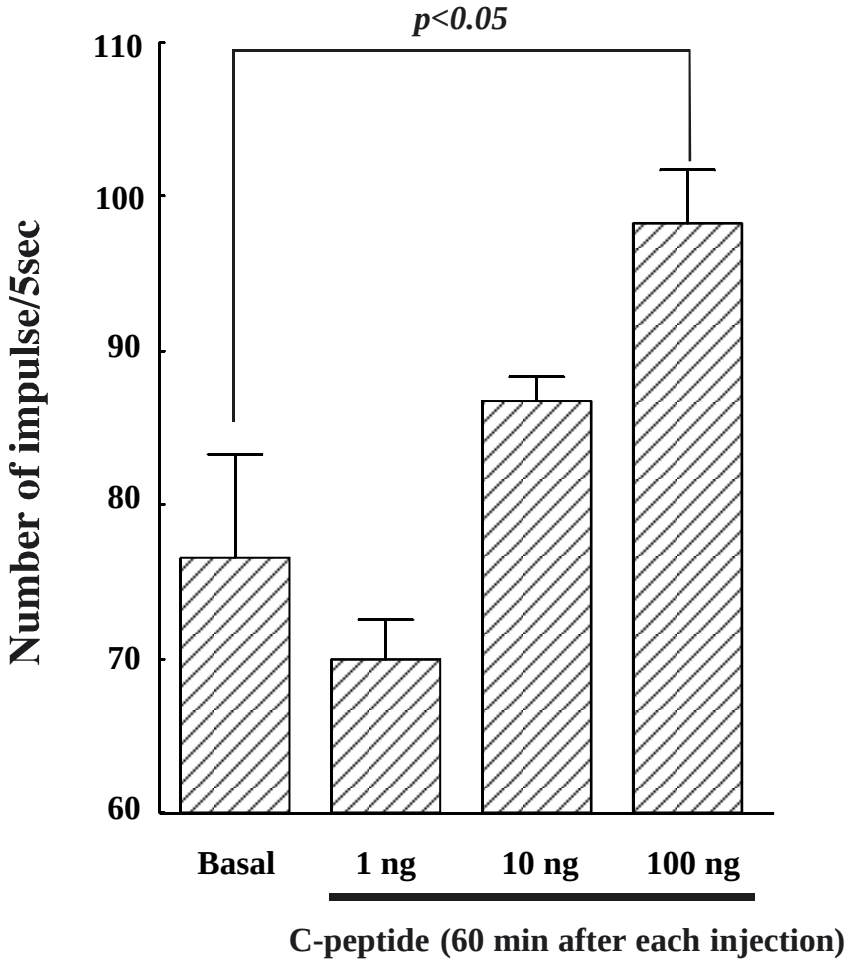
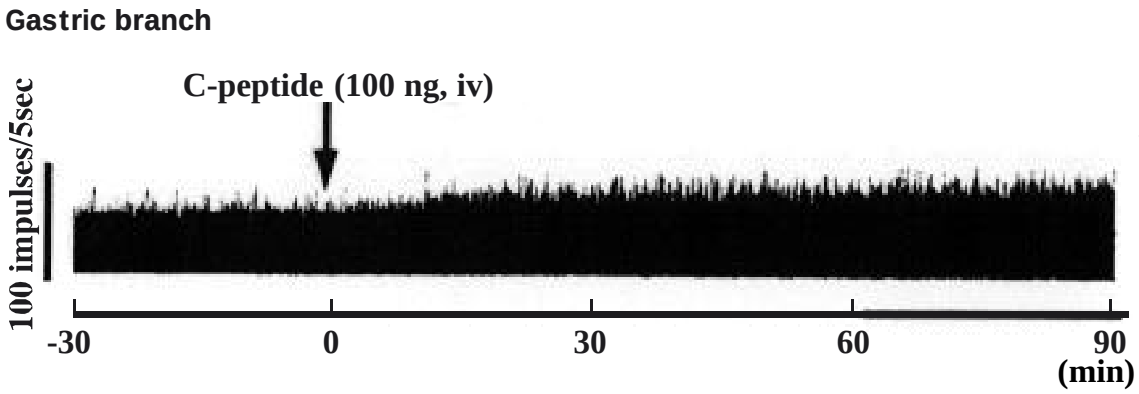

p<0.05
110
100
90
80
70
60
Number of impulse/5sec
Basal
1 ng
10 ng
100 ng
C-peptide (60 min after each injection)

Fig. 1B


Gastric branch
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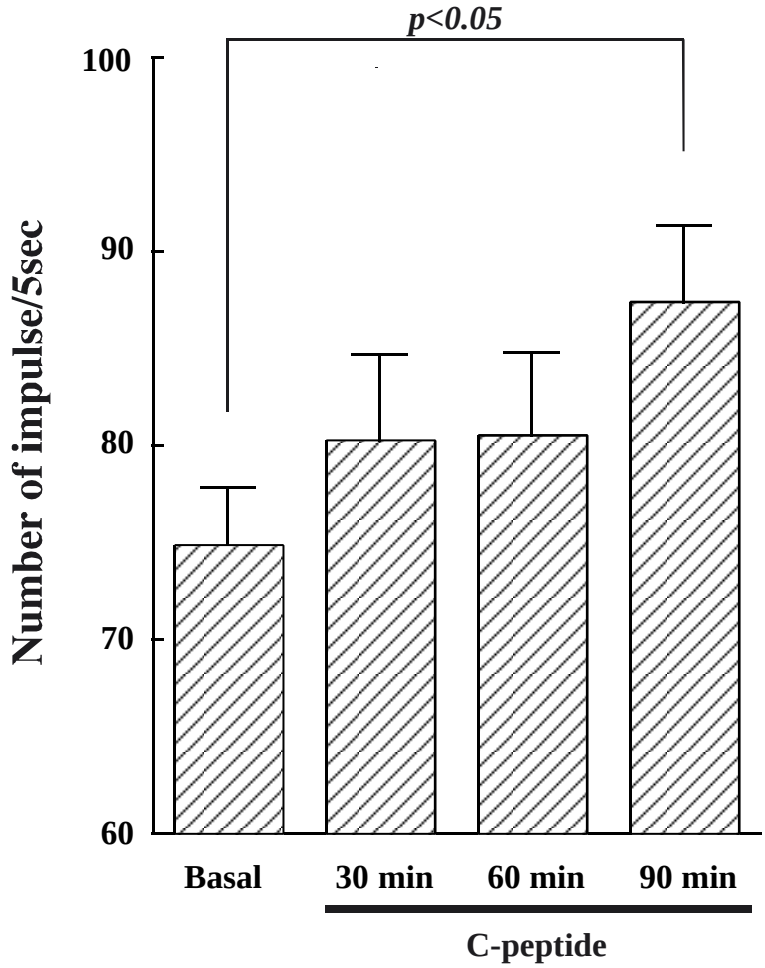
-30
0
30
60
90
(min)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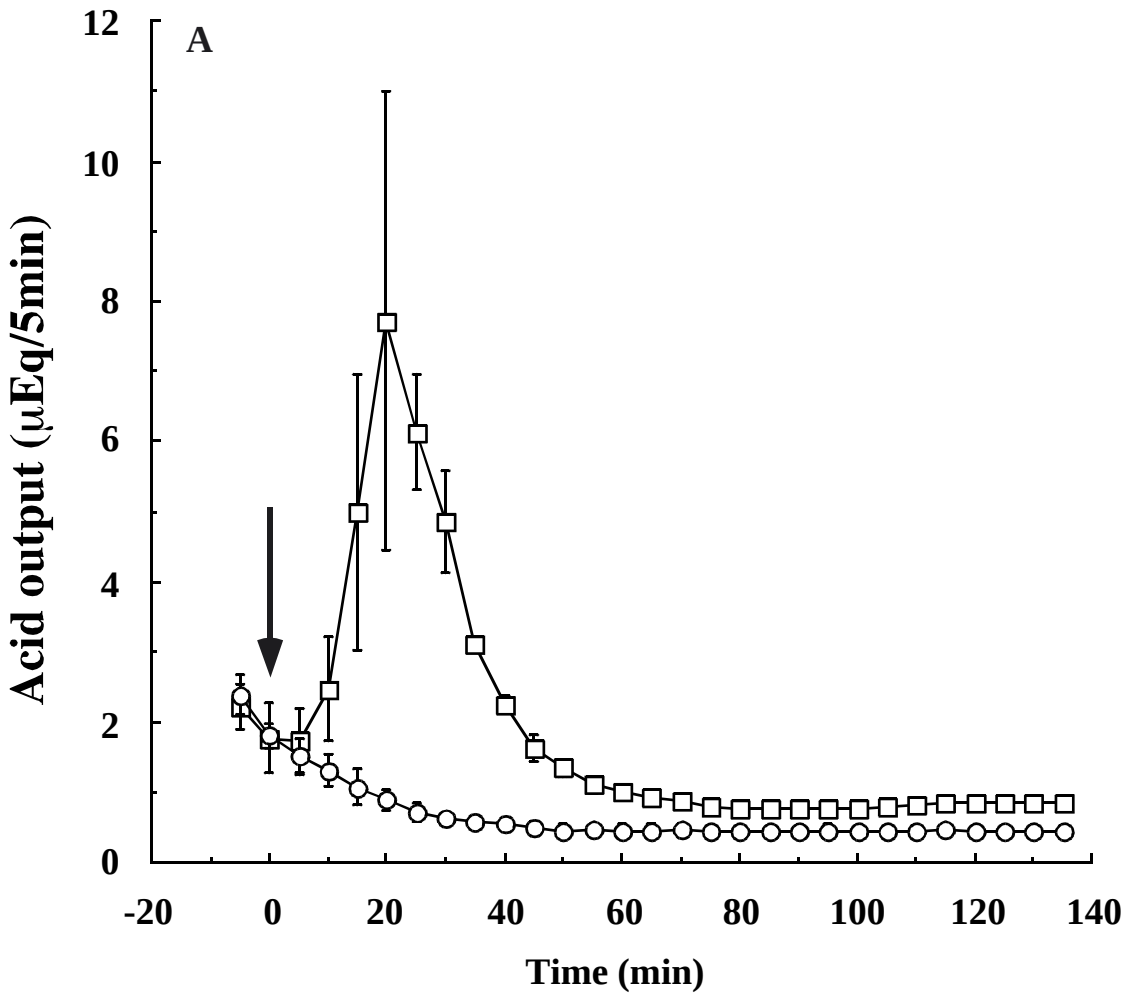
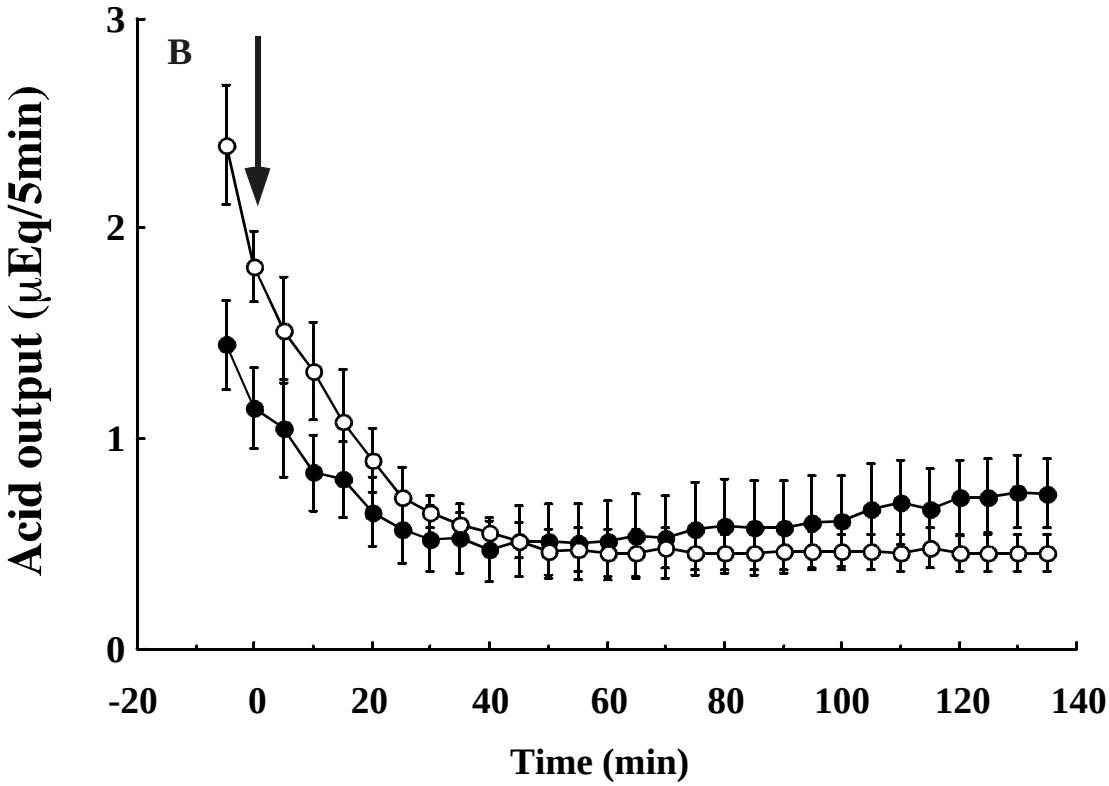
90
80
70
60
Number of impulse/5sec
Basal
30 min
60 min
90 min
C-peptide

Fig.2


A
Acid output (μEq/5min)
12
10
8
6
4
2
0
-20
0
20
40
60
80
100
120
140
Time (min)


B
Acid output (μEq/5min)
3
2
1
0
-20
0
20
40
60
80
100
120
140
Time (min)

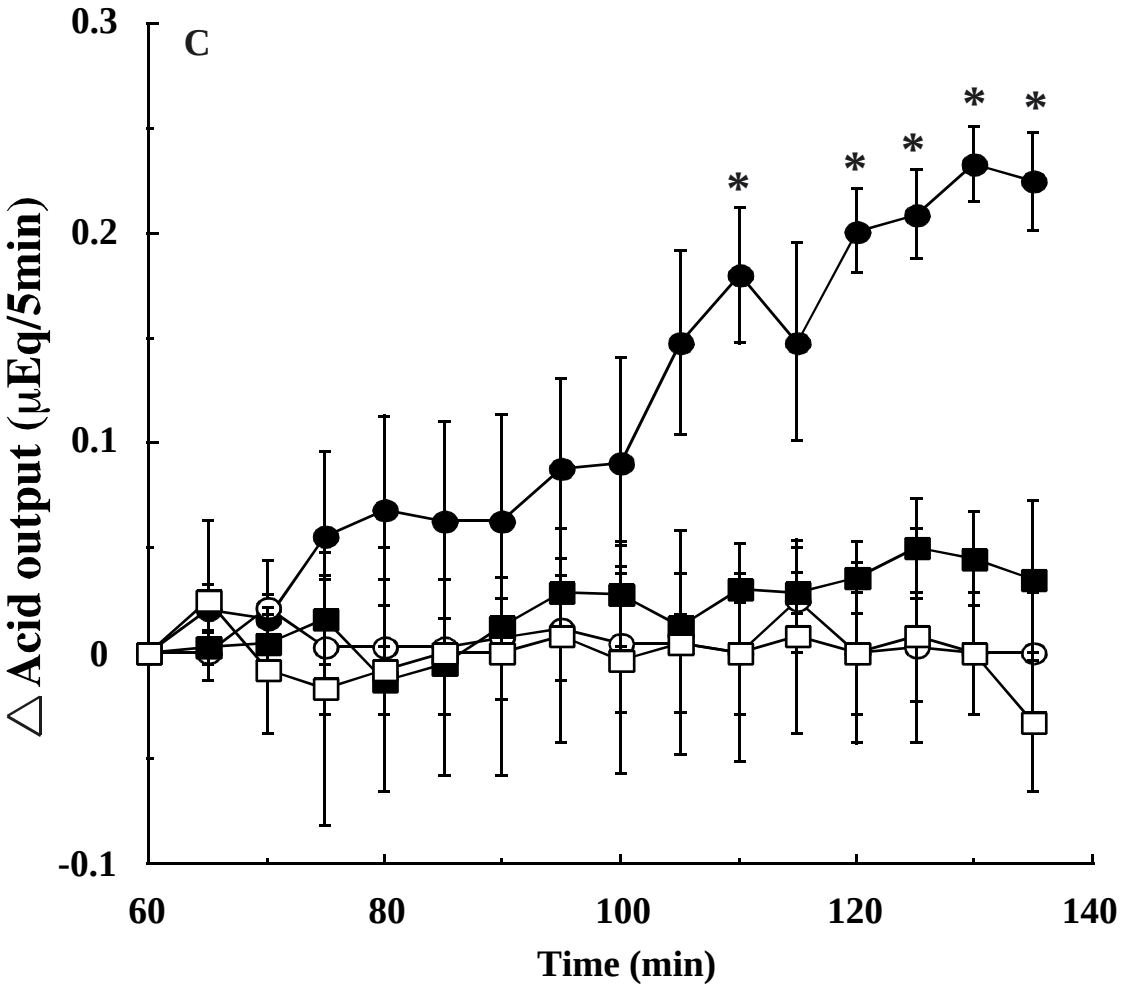



Fig.2 (continued)