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The Increases of Proenkephalin A mRNA Levels and the Secretion of [Met^5]-Enkephalin Induced by Long-term Stimulation with Nicotine are Mediated by a Lipoxygenase Pathway in Bovine Adrenal Medullary Chromaffin Cells

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ABSTRACT

The effect of nicotine on the secretion of [Met^5]-enkephalin (ME) in addition to proenkephalin A (proENK) mRNA levels and effects of indomethacin, nordihydroguaiaretic acid (NDGA), and captopril on nicotine-induced responses were studied in bovine adrenal medullary chromaffin (BAMC) cells. Long-term exposure of BAMC cells to nicotine at a concentration of 10 μM significantly increased proENK mRNA level and the secretion of ME into the medium. Treatment of BAMC cells with NDGA (a lipoxygenase inhibitor, 10 μM), indomethacin (a cycloooxygenase inhibitor) or captopril (an angiotensin converting enzyme inhibitor) alone did not affect ME secretion and proENK mRNA levels. The pretreatment of BAMC cells with NDGA inhibited the increased ME secretion and proENK mRNA level induced by nicotine. However, indomethacin and captopril did not affect nicotine-induced responses. Our results indicate that neuronal regulations of ME secretion and proENK mRNA level induced by nicotine in BAMC cells are in part mediated by a lipoxygenase-but not cyclooxygenase-and endogenous renin-angiotensin pathway.

Key Words: Nicotine, Indomethacin, Proenkephalin A, Met-enkephalin, Nordihydroguaiaretic acid, Captopril

INTRODUCTION

[Met^5]-enkephalin (ME) in adrenal chromaffin cells is co-stored and co-released with catecholamines (Viveros *et al.*, 1979; Livett *et al.*, 1981). The secretion of ME or catecholamines, as well as the expression of proenkephalin A (proENK) or tyrosine hydroxylase (TH) mRNA, can be regulated by activity of the splanchnic nerve, which releases acetylcholine as the major neurotransmitter in the adrenal medulla. The existence of nicotinic receptors on bovine adrenal medullary chromaffin (BAMC) cells have raised the possibility that the cholinergic neural input to the adrenal medulla regulates ME and catecholamine secretion as well as the expression of the corresponding genes. The effects of nicotine on ME and catecholamine secretion have been investigated both in vivo and in vitro (Harish *et al.*, 1987; Khalil *et al.*, 1988; Watkinson *et al.*, 1990; Wilson *et al.*, 1982). In addition, the neural regulation of the proENK and TH genes in the adrenal medulla has been studied using stress or insulin-induced hypoglycemia (Kanamatsu *et al.*, 1986; Stachowiak *et al.*, 1985; Weisinger *et al.*, 1990) as well as *in vitro* using nicotine (Eiden *et al.*, 1984; Stachowiak *et al.*, 1990, 1991).

Several lines of evidence have demonstrated that arachidonic acid (AA) in BAMC cells is re-

leased into the medium by various types of stimulators including nicotine (Fyre and Holz, 1984; Morgan and Burgoyne, 1990). *Cis*-unsaturated fatty acid can be liberated by phopholipase A_2 or diacylglycerol lipase and serve as a precursor, and subsequently metabolized further via the action of cyclooxygenase and lipoxygenase (Burgoyne *et al.*, 1987; Murphy and Pearce, 1988). Short-term stimulation of BAMC cells with AA had either no or only a small effect on the secretion of catecholamine or ME from BAMC cells (Suh *et al.*, 1992a; Marley *et al.*, 1988; Yokohama *et al.*, 1988a, b; Tanaka *et al.*, 1990; Negishi *et al.*, 1990). However, we recently reported that prolonged stimulation of BAMC cells with AA increases expression of the proENK gene and the secretion of ME (Suh *et al.*, 1992a).

The existence of high density of angiotensin II receptors in the adrenal medulla has recently been reported (Himeno *et al.*, 1988). Recently we and other have reported that continuous activation of angiotensin II receptors by [Sar^1] angiotensin II increases expression of proENK mRNA in BAMC cells (Suh *et al.*, 1992b; Wan *et al.*, 1990a, b; Stachowiak *et al.*, 1991). In addition, angiotensin II was found to increase the secretion of ME into the culture medium after long-term (24 hr) but not short-term (30 min) stimulation, suggesting that angiotensin II in BAMC cells exerts a major effect on the long-term regulation of expression of proENK mRNA and secretion of ME. Furthermore, we have previously reported that there exists an endogenous renin-angiotensin system in BAMC cells (Poisner *et al.*, 1992). However, the role of endogenous renin-angiotensin system in BAMC cells has not been well characterized.

In recent studies, we and others have reported that long-term stimulation of BAMC cells with nicotine increases proENK and TH mRNAs in BAMC cells (Suh *et al.*, 1992c; Stachowiak *et al.*, 1990, 1991). In addition, we found in previous study that nicotine increases both the short-and long-term secretion of ME (Suh *et al.*, 1992c). Therefore, the present study was designed to determine if nicotine-induced increases of ME secretion and proENK mRNA level are mediated by an AA cascade system or an endogenous renin-angiotensin system in BAMC cells. Effects of indomethacin (a cyclooxygenase inhibitor), NDGA (a lipoxygenase inhibitor), or captopril (an angiotensin converting enzyme inhibitor) on nicotine-induced responses were examined.

MATERIALS AND METHODS

Chemicals

Nicotine HCl, indomethacin, nordihydroguaiaretic acid, and captopril were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture

Primary cultures of BAMC cells were prepared according to the method of Wilson (1987). Briefly, bovine adrenal glands were retrogradely perfused with collagenase (3×15 min, 37℃) and medullae were dissected and further digested in a trypsinizing flask (20 min, 37℃). Then the BAMC cells were isolated by Renografin gradient centrifugation. The cells were mixed into DMEM/F12 medium containing 10% fetal calf serum and plated on 30-mm wells (5×10^6 cells/well in 5 ml of medium) and incubated in an humidified air/5% CO_2-atmosphere at 37℃. Two days after plating, the cells were incubated with serum-free medium. To avoid variations between separate cell preparations, all the experiments were performed 24 hr after the medium was changed with serum-free medium.

Radioimmunoassay

The levels of ME-like immunoreactivity were determined by radioimmunoassay (RIA) as described by Hong *et al.* (1978) and Suh *et al.* (1992d). Samples were assayed in duplicate. Iodinated ME (10,000 cpm) was incubated overnight at 4℃ with various concentrations of the ME standard, medium or cellular extracts, and the ME rabbit antiserum in a final volume of 0.5 ml. Separation of bound ME from free ME was accomplished by incubation with 0.2 ml of a charcoal slurry containing 2% bovine serum albumin (BSA) in RIA solution for 20 min and centrifugation at 4,000 xg for 10 min. Five hundred μl of the supernatant were counted for radioactivity.

The antiserum to ME was produced by the method of Hong *et al.* (1978) by immunization of rabbits with ME conjugated to thyroglobulin and

had the following cross-reactivities: ME, 100%; [Met-O^5]enkephalin, 80%; [Leu^5]-enkephalin, <1 %; [Met^5]-enkephalin-Arg^6-Phe^7, <0.4%; dynorphin (1~8), 0.06%; and β-endorphin, <0.2%.

Isolation of RNA and mRNA assay

Total RNA was extracted from BAMC cells in a single step by a guanidinium isothiocyanate/phenol/chloroform gradient procedure and the relative abundance of proENK mRNA was assayed by Northern blot analysis (Chomczynski and Sacchi, 1987). Ten μg of RNA were denatured and electrophoresed on a 1.2% agarose gel and transferred to nylon hybond-N hybridization filter sheets (Amersham). After baking for 2 hr at 80℃, transfer membranes were prehybridized at 42℃ for at least 4 hr. The radiolabeled proENK probe was added and the membrane was incubated overnight (>14 hr) at 42℃. Following hybridization, the membranes were washed three times with 2X standard saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) at 42℃ for 20 min. The membranes were then dried and exposed to Kodak XAR-5 film at −70℃ for 1~3 days. The intensities of the hydridized bands were quantified by scanning densitometry with a Gilford densitometer. After hybridization with the proENK probe, membranes were rehybridized with a radiolabeled β-actin probe as an internal standard.

To quantify the abundance of proENK, synthetic antisense oligonucleotide was used as a probe. The sequence of the nucleotides for proENK was 5'-GCC GAG CGC CAG CAG CCA AGT GCA GAG TCC CAG GAA CCG CGC-3' which consists of the 2nd to the 14th codons (Noda *et al.*, 1982).

Experimental procedure

To minimize variations between separate cell preparations, all the experiments were performed 24 hr after the medium was changed to a serum-free medium. BAMC cells were pretreated with either indomethacin (10 μM), NDGA (10 μM) or captopril (10 μM) for 15 min followed by nicotine (10 μM) for 24 hr. The cells were harvested 24 hr after nicotine treatment.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test for overall statistical significance. Multiple comparisons between groups were made using Fisher's Least Significance Test (Miller, 1966).

RESULTS

Effect of nicotine on the secretion of ME and proENK mRNA

Long-term exposure (24 hr) of BAMC cells to nicotine (10 μM) increased secretion of ME and proENK mRNA level as shown in Figs. 1 and 2.

Effects of indomethacin and NDGA on the secretion of ME and the expression of proENK mRNA induced by nicotine

In order to determine if metabolites of AA are involved in the long-term (24 hr) action of nicotine, BAMC cells were pretreated with either a cyclooxygenase inhibitor (indomethacin) or a lipoxygenase inhibitor (NDGA). Neither 10 μM indomethacin nor 10 μM NDGA treated alone caused a change in either ME secretion or proENK mRNA expression in control cultures. Pretreatment with NDGA partially inhibited the

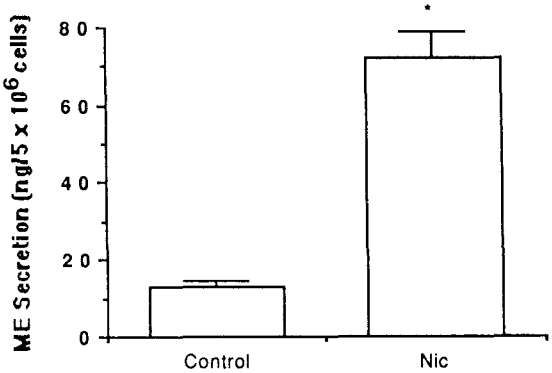

Fig. 1. Nicotine increases the secretion of ME. The BAMC cells (5×10^6 cells/well) were treated with 10 μM nicotine (Nic) for 24 hr. The ME levels were measured by a radioimmunoassay. The veritical bars indicate the standard error of the mean (*, $p<0.05$ compared to the control group; n=3 independent experiments with triplicate samples).

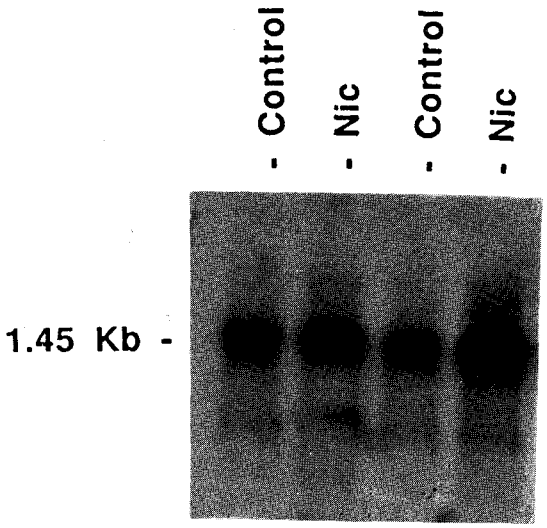

Fig. 2. Nicotine increases the expression of the proENK mRNA. The BAMC cells (5×10^6 cells /well) were treated with 10 μM nicotine (Nic) for 24 hr. Total RNA was prepared using a guanidinium / isocyanate / phenol / chloroform gradient method. Ten μg of total RNA were used for the determination of the proENK mRNA levels by Northern blot analysis.

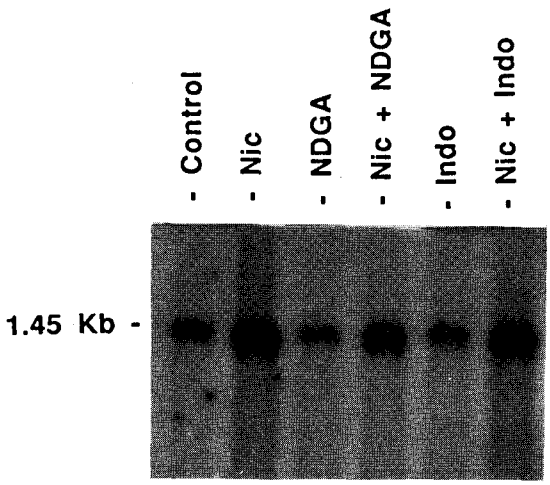

Fig. 4. Effects of pretreatment with NDGA and indomethacin on proENK mRNA levels induced by nicotine. BAMC cells (5×10^6 cells/well) were pretreated with NDGA (10 μM) or indomethacin (Indo, 10 μM) for 15 min and the cells were incubated at 37℃ with nicotine (Nic, 10 μM) for 24 hr.

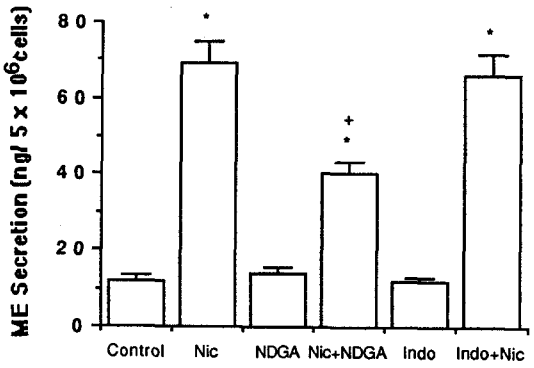

Fig. 3. Effects of NDGA and indomethacin on ME secretion induced by nicotine. BAMC cells (5×10^6 cells/well) were pretreated with NDGA (10 μM) or indomethacin (Indo, 10 μM) for 15 min and the cells were incubated at 37℃ with nicotine (Nic, 10 μM) for 24 hr. The vertical bars indicate the standard error of the mean (*, $p<0.05$ compared to the control group; +, $p<0.05$ compared to the nicotine-treated group; n=3 independent experiments with triplicate samples).

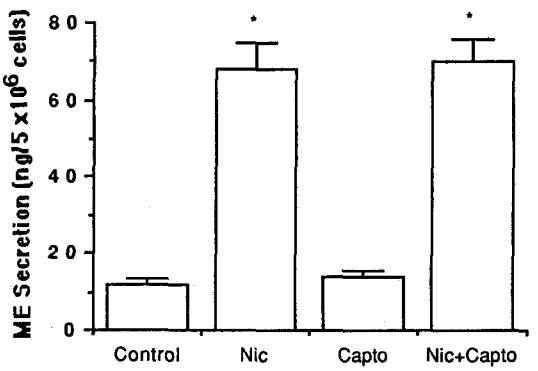

Fig. 5. Captopril has no effect on increased ME secretion induced by nicotine. BAMC cells (5×10^6 cells/well) were pretreated with captopril (Capto, 10 μM) for 15 min and the cells were incubated at 37℃ with nicotine (Nic, 10 μM) for 24 hr. The vertical bars indicate the standard error of the mean. (*, $p<0.05$ compared to the control group; n=3 independent experiments with triplicate samples).

secretion of ME and expression of proENK mRNA induced by long-term nicotine (Fig. 3 and 4). However, indomethacin did not alter nicotine-induced responses (Fig. 3 and 4).

Effect of captopril on the secretion of ME and expression of proENK mRNA induced by nicotine

To determine if an endogenous renin-angiotensin system was involved in the increases of ME secretion and proENK mRNA levels induced by nicotine, the effect of captopril (an angiotensin converting enzyme inhibitor) on nicotine-induced responses were examined. Ten μM captopril did not make a change in either ME secretion or proENK mRNA expression in control cultures (Figs. 5 and 6). Pretreatment with captopril did not affect the increased secretion of ME and expression of proENK mRNA induced by long-term nicotine (Figs. 5 and 6).

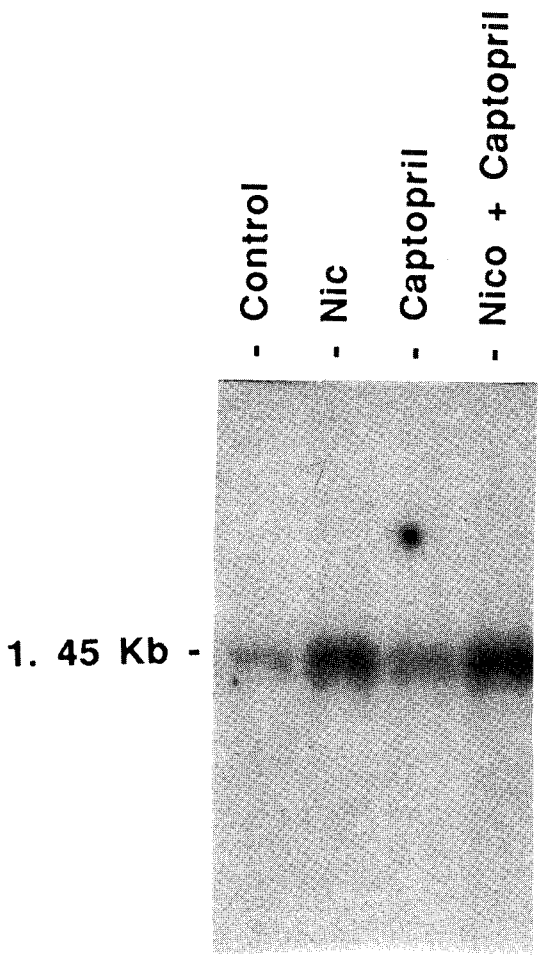

Fig. 6. Captopril has no effect on increased proENK mRNA levels induced by nicotine. BAMC cells (5×10^6 cells/well) were pretreated with captopril (10 μM) for 15 min and the cells were incubated at 37℃ with nicotine (Nic, 10 μM) for 24 hr.

DISCUSSION

The present study demonstrated that the continuous stimulation up to 24 hr of nicotinic receptors of BAMC cells increased both the long-term secretion of ME and proENK mRNA levels. In the previous study, we have reported that nicotine clearly increases transcripitional rate of the proENK gene (Suh *et al.*, 1992c). In addition, the continuous stimulation up to 6 hr of nicotinic receptors is required for both the long-term secretion of ME and proENK mRNA levels. We also have reported that newly-synthesized ME is the major component of the long-term secretion of ME for nicotine, as reported in our previous studies for angiotensin II and other secretagogues (Suh *et al.*, 1992a, b, and d, 1993).

The presence of AA and PGE_2 receptors in BAMC cells has raised the possibility that they play a role in the regulation of medullary hormone secretion as well as gene expression. We have recently reported that both AA and PGE_2 regulate the long-term secretion of ME as well as proENK mRNA level in BAMC cells. This contention was supported by the finding that short-term stimulation (1 hr) of BAMC cells with AA increased the secretion of ME to only a small extent, whereas long-term AA treatment caused a profound increase in the secretion of ME (Suh *et al.*, 1992a). Morgan and Burgoyne (1990) reported that AA can be liberated by calcium or nicotine via a diacylglycerol pathway. Although it is controversial whether AA is involved in short-term secretion, the present study clearly demonstrated that AA metabolites are involved in the long-term regulation of both ME secretion and proENK mRNA expression. In support of this finding, we found that NDGA, a lipoxygenase inhibitor (Ramwell *et al.*, 1966), effectively inhibited both

ME secretion and the proENK mRNA level induced by nicotine, suggesting that the nicotine-induced responses are mediated by a lipoxygenase pathway. However, indomethacin, a cyclooxygenase inhibitor, had no effect on nicotine-induced responses, indicating that a cyclooxygenase pathway is probably not involved in nicotine-induced responses. We are currently investigating which metabolites of the lipoxygenase pathway are involved in nicotine-induced responses.

During the last several years of period, many lines of evidence that angiotensin II is an important hormonal regulator for the long-term secretion of ME and the expression of proENK mRNA have been accumulated. We have recently demonstrated that angiotensin II has pronounced effect on long-term regulation of the biosynthesis and secretion of ME but has no significant short-term effect in BAMC cells (Suh *et al.*, 1992b). In addition to this hormonal regulation of angiotensin II, there exist an endogenous renin-angiotensin system in BAMC cells. This contention is supported by our previous study that stimulators of adenyl cyclase or protein kinase C caused increase in renin and prorenin in the incubation medium and the BAMC cells, suggesting that an autocrine or paracrine mechanism involving the endogenous renin-angiotensin system for regulating of chromaffin cells function (Poisner *et al.*, 1992). However, in the present study, we found that pretreatment with captopril, an angiotensin converting enzyme inhibitor, did not alter the increased ME secretion and proENK mRNA level induced by nicotine, indicating that an endogenous renin-angiotensin system is not involed in nicotine-induced responses.

We conclude that nicotine exerts a pronounce effect on proENK gene expression and long-term secretion in the adrenal medulla. These effects appear to be mediated in part by a lipoxygenase-but not a cyclooxygenase-and an endogenous renin-angiotensin pathway.

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=국문초록=

소 부신수실 크롬친화성 세포에서 Nicotine의 장기간 자극으로 유발된 Proenkephalin A mRNA의 증가 및 [Met^5]-enkephalin의 분비 증가가 Lipoxygenase 경로에 의해 매개됨

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서 홍 원·김 영 희

소 부신수실 크롬친화성 세포(BAMC)에서 [Met^5]-enkephalin (ME)의 분비와 Proenkephalin A (proENK) mRNA의 함량에 대한 nicotine의 영향과 이에 대한 indomethacin, nordihydroguaiaretic acid(NDGA) 및 captopril의 작용을 연구하였다. 10 uM Nicotine으로 BAMC 세포를 장기간 자극시 proENK mRNA의 함량과 배양액으로의 ME 분비가 유의하게 증가하였다. BAMC 세포를 NDGA(lipoxygenase 억제제, 10 uM), indomethacin (cyclooxygenase 억제제), captopril (angiotensin 변환효소 억제제)만으로 처리시에는 ME 분비와 proENK mRNA의 함량에 영향이 없었다. Nicotine에 의한 ME 분비와 proENK mRNA 함량의 증가는 NDGA에 의하여 억제되었다. 그러나 indomethacin과 captopril은 nicotine의 작용에 대하여 아무 영향이 없었다. 이들 결과는 nicotine에 의한 ME 분비와 proENK mRNA 함량의 증가가 부분적으로 lipoxygenase 경로에 의해 매개되며, cyclooxygenase 및 내재성 renin-angiotensin 경로는 관련되지 않음을 나타낸다.