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Mechanism of Leptin-Induced Potentiation of Catecholamine Secretion Evoked by Cholinergic Stimulation in the Rat Adrenal Medulla

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The aim of the present study was to examine the effect of leptin on CA release from the isolated perfused model of the rat adrenal gland, and to establish its mechanism of action. Leptin (1~100 ng/ml), when perfused into an adrenal vein of the rat adrenal gland for 60 min, enhanced a dose-dependently the secretory responses of CA evoked by ACh (5.32×10^{-3} M), DMPP (10^{-4} M) and McN-A-343 (10^{-4} M), although it alone has weak effect on CA secretion. However, it did not affect the CA secretion evoked by excess K^+ (5.6×10^{-2} M). Leptin alone produced a weak secretory response of the CA. Moreover, leptin (10 ng/ml) in to an adrenal vein for 60 min also augmented the CA release evoked by BAY-K-8644, an activator of the dihydropyridine L-type Ca^{2+} channels, and cyclopiazonic acid, an inhibitor of cytoplasmic Ca^{2+} ATPase. However, in the presence of U0126 (1 μM), an inhibitor of mitogen-activated protein kinase (MAPK), leptin no longer enhanced the CA secretion evoked by ACh and DMPP. Furthermore, in the presence of anti-leptin (10 ng/ml), an antagonist of Ob receptor, leptin (10 ng/ml) also no longer potentiated the CA secretory responses evoked by DMPP and Bay-K-8644. Collectively, these experimental results suggest that leptin enhances the CA secretion from the rat adrenal medulla evoked by cholinergic stimulation (both nicotininc and muscarinic receptors), but does not that by membrane depolarization. It seems that this enhanced effect of leptin may be mediated by activation of U0126-sensitive MAPK through the leptin receptors, which is probably relevant to the activation of the dihydropyridine L-type Ca^{2+} channels located on the rat adrenomedullary chromaffin cells.

Key Words: Leptin, Catecholamine release, Adrenal medulla, Leptin receptors, MAPK pathway

INTRODUCTION

Leptin, the protein product of the cloned obese gene (Zhang et al, 1994) has been recognized as another mediator of energy expenditure. Leptin is secreted from adipose tissues and acts through the hypothalamus, the center of energy homeostasis (Zhang et al, 1994; Taltaglia et al, 1995; Friedman & Halaas 1998). Several lines of evidence have shown that in addition to hypothalamic actions, leptin acts directly on peripheral tissues, such as the pancreatic islets (Pallett et al, 1997), hematopoietic cells (Gainsford et al, 1996) or the T-lymphocytes (Lord et al, 1998) and the reproductive system (Barash et al, 1996). Indeed, the mRNA of leptin receptors (ObRs) is expressed not only in the hypothalamus, but also in various peripheral tissues (Tanaglia et al, 1995; Lee et al, 1996). ObRs are assumed to activate several signal transduction pathways, including the mitogen-activated protein kinase (MAPK) cascade (Takahashi et al, 1997; Tanabe et al, 1997) and the Janus protein-tyrosine kinase (JAK)-signal transducers and activators of transcription (STAT) cascade (Rosenblum et al, 1996). Moreover, it has been shown that leptin stimulates synthesis of catecholamines (CA) in cultured bovine adrenal medullary cells via the activation of tyrosine hydroxylase by two different mechanisms, i.e., one is dependent on tyrosine hydroxylase phosphorylation mediated through the MAPK pathway and the second is independent of enzyme phosphorylation (Utsunomiya et al, 2001). CAs are neurotransmitters or hormones that can cause weight loss via a number of mechanisms, including lipolysis, the inhibition of insulin secretion, increased blood glucose and thermogenesis. The evidence has shown that leptin increases CA secretion from the adrenal medulla via stimulation of the central sympathetic nervous system (Satoh et al, 1999). In contrast, CA and β-adrenergic receptor agonists inhibit the expression, synthesis and secretion of leptin (Kosaki et al, 1996; Evans et al, 1999). From these data, it is tempting to speculate that leptin and CA might directly or indirectly interact with each other to control metabolic homeostasis. The presence of the ObR mRNA or

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ABBREVIATIONS: CA, catecholamine; ObRs, leptin receptors; ACh, acetylcholine; DMPP, 1.1-dimethyl-4-phenyl piperazinium iodide; MAPK, mitogen-activated protein kinase; JAK, Janus protein-tyrosine kinase; STAT, signal transducers and activators of transcription.

its protein has been reported in the rat (Cao et al, 1997), murine (Hoggard et al, 1997), human (Glasow et al, 1998) and porcine (Takekoshi et al, 1999) adrenal medulla. However, there are conflicting reports on the function of ObR; leptin either has no effect on (Glasow et al, 1998) or stimulates (Takekoshi et al, 1999) CA secretion. ObR and its mRNA in the bovine adrenal medulla by I25I-leptin binding and the reverse transcriptase-polymerase chain reaction (RT-PCR) are also identified and characterized, respectively (Yanagihara et al, 2000). Furthermore, it has been found that stimulation of ObR by leptin increased the synthesis of ^{14}C-CA in the cells.

Therefore, the present study was designed to examine the effect of leptin on CA release from the isolated perfused model of the rat adrenal gland, and to establish its mechanism of action.

METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180~300 g, were anesthetized intraperitoneally with thiopental sodium (40 mg/kg). The adrenal gland was isolated by the methods described previously (Wakade, 1981). The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by the placement of three-hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads, and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations. A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Before ligating vessels and cannulations, heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation. A small slit was made into the adrenal cortex just opposite entrance of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. The adrenal gland, along with ligated blood vessels and the cannula, was then carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at $37\pm1^{\circ}C$.

Perfusion of adrenal gland

The adrenal glands were perfused by means of a peristaltic pump (WIZ Co.) at a rate of 0.3 ml/min in rats and 0.8 ml/min in rabbits. The perfusion was carried out with Krebs-bicarbonate solution of following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The solution was constantly bubbled with 95 % O_2+5% CO_2, and the pH of the solution was maintained at 7.4~7.5. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of CAs.

Drug administration

The perfusions of DMPP (100 μM) and McN-A-343 (100 μM) for 2 minutes, and Bay-K-8644 (10 μM) for 4 minutes were made into perfusion stream, respectively. A single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 ml was injected into perfusion stream via a three-way stopcock, respectively.

In the preliminary experiments, it was found that, upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to pre-injection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

Prior to stimulation with various secretagogues, perfusate was routinely collected for 4 min to determine spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, the perfusates were continuously collected in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated samples were collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from that secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures. To study the effects of leptin on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing leptin for 60 min immediately after the perfusate was collected for a certain minute (background sample). And the medium was then changed to the one containing the stimulating agent, and the perfusates were collected for the same period as that for the background sample. Generally, the adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

CA content of perfusate was fluorospectrophotometrically (Kontron Co. Italy) measured directly by the fluorometric method of Anton and Sayre (1962) without intermediate purification on alumina for the reasons described earlier (Wakade, 1981). A volume of 0.2 ml perfusate was used for the reaction. The CA content in the glands perfusate stimulated by secretogagues in the present work was high enough to obtain several folds greater readings than that of control samples (unstimulated). The sample blanks were also the lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Statistical analysis

The statistical significance between groups was determined by utilizing the Student's t-test. A P-value of less than 0.05 was considered to represent statistically significant changes, unless specifically noted in the text. Values given in the text refer to means and standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

Drugs and their sources

Leptin, antileptin, U0126, acetylcholine chloride (ACh), 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, ethyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-2-rifluoromethylphenyl)-pyridine-5-carboxylate (BAY-8644) and cyclopiazonic acid were purchased from Sigma Chemical Co., U.S.A. 4-(N-[3-Chlorophenyl]carbamoyloxy)-2-butynyltrimethyl ammonium chloride (McN-A-343) was purchased from RBI Co., U.S.A. Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644, which was dissolved in 99.5% ethanol and diluted appropriately (final concentration of alcohol was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

The effect of leptin on the CA secretion

When the adrenal gland was perfused with oxygenated Krebs-bicarbonate solution for 60 min before experimental protocol is initiated, the spontaneous CA secretion reached steady state. The basal CA release from the perfused rat adrenal medulla amounted to 20±2 ng for 2 min from 8 experiments. The releasing effects to the initial perfusion of leptin (10^{-8} M) for 60 min are shown in Fig. 1. Time-course effect of leptin (10^{-8} M) infusion into the perfusion stream for 60 min produced the significant responses of CA secretion over the background release, leading to the peak secretion at 40~60 min period. This result seems to be similar to the findings that leptin directly stimulates catecholamine secretion and synthesis in cultured porcine adrenal medullary chromaffin cells (Takekoshi et al, 1999).

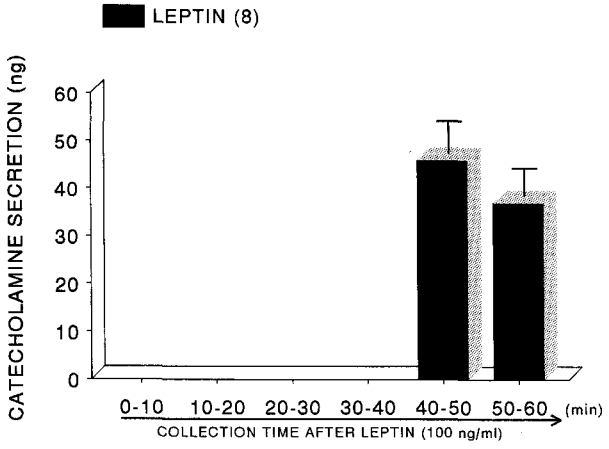

Fig. 1. Time course effect of continuous infusion of leptin on the secretion of catecholamines (CA) from the isolated perfused rat adrenal glands. Leptin (100 ng/ml) was perfused into an adrenal vein for 60 min as indicated at an arrow mark. Numbers in the parenthesis indicate number of rat adrenal glands. The vertical columns and bars denote the means and the standard errors of the corresponding means, respectively. Ordinate: the amounts of CA secreted from the adrenal gland (ng). Abscissa: collection time of perfusate (min). Leptin-induced perfusates were collected for 60 min at 10 min intervals.

In 8 rat adrenal glands, this leptin -evoked CA secretory responses were 21~64 ng (0~60 min), which seemed to be a very weak secretagogue. The tachyphylaxis to releasing effects of CA evoked by leptin was not observed (data not shown). However, in all subsequent experiments, leptin was not administered more than twice only at 120 min interval.

Effect of leptin on CA secretion evoked by ACh, high K^+, DMPP, and McN-A-343 from the perfused rat adrenal glands

In order to examine the effects of leptin on CA release, the dose-dependent effect of leptin on CA seretory responses evoked by ACh and high potassium was examined. As illustrated in Fig. 2 and 3, 100 ng/ml of leptin concen-

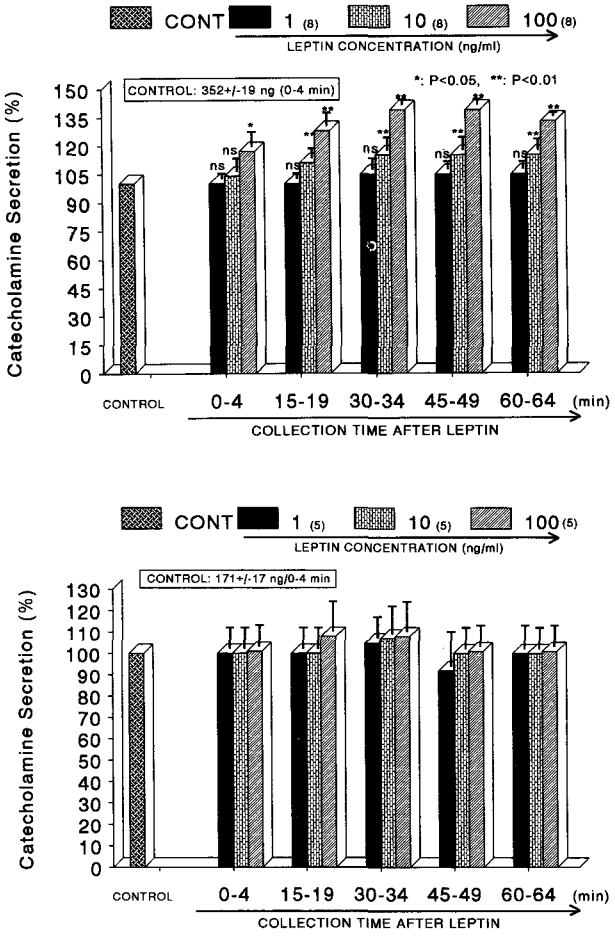

Fig. 2. Effects of leptin on the secretory responses of catecholamines evoked by acetylcholine (upper) and high potassium (lower) from the isolated perfused rat adrenal glands. CA secretion by a single injection of Ach (5.32×10^{-3} M) or high K^+ (5.6×10^{-2} M) was induced 'BEFORE (CONTROL)' and 'AFTER' preloading with leptin (1~100 nM) for 60 min. Statistical difference was obtained by comparing the corresponding 'CONTROL' with each period 'AFTER' the intiation of leptin perfusion. Perfusates were collected for 4 minutes at 15 min intervals. Other legends are the same as in Fig. 1. High K^+-induced CA release was not affected by treatment with leptin. ns: Statistically not significant.

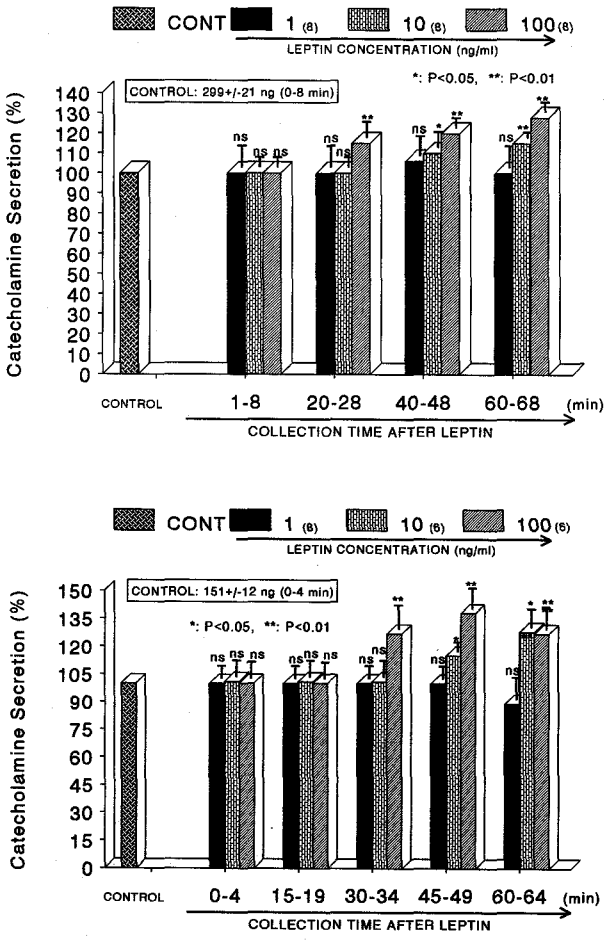

Fig. 3. Effects of leptin on the secretory responses of catecholamines evoked by DMPP (upper) and McN-A-343 (lower) from the isolated perfused rat adrenal glands. CA secretion by perfusion of DMPP (10^{-4} M) or McN-A-343 (10^{-4} M) was induced before (CONTROL) and after perfusion with leptin (1~100 nM) for 60 min. DMPP- and McN-A-343-induced perfusates were collected for 8 and 4 minutes at 20 and 15 min interval, respectively. Other legends are the same as in Fig. 1. ns: Statistically not significant.

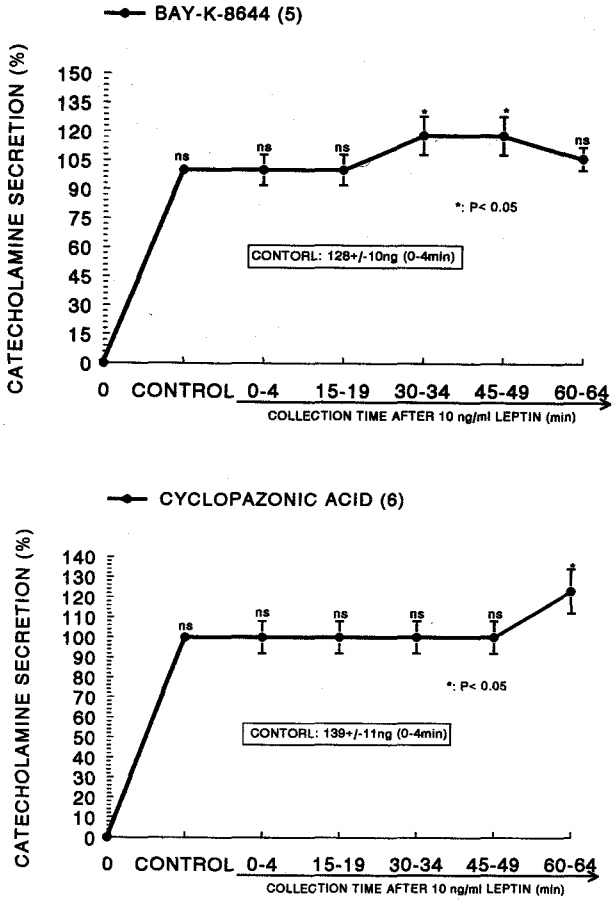

Fig. 4. Effects of leptin on the secretory responses of catecholamines evoked by Bay-K-8644 and cyclopiazonic acid from the isolated perfused rat adrenal glands. CA secretion by perfusion of Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) for 4 min was induced before (CONTROL) and after perfusion with leptin (10 nM) for 60 min, respectively. Bay-K-8644 -induced perfusates were collected for 4 minutes at 15 min interval. Other legends are the same as in Fig. 1. ns: Statistically not significant.

trations produced effective enhancement of CA secretory responses evoked by ACh, DMPP and McN-A-343, not by high K^+. In the present experiment, ACh (5.32×10^{-3} M)-evoked CA release prior to the perfusion with leptin was 352 ± 19 ng (0~4 min). In the presence of leptin (1~100 ng/ml) for 60 min, it was significantly increased by 139% at 30~49 min, but in the presence of low leptin (1 ng/ml) it was never affected in comparison to the corresponding control (Fig. 2-upper). Also, KCl, a direct membrane-depolarizing agent, sharply stimulates CA secretion. In the present work, high K^+ (5.6×10^{-2} M)-evoked CA release in the presence of leptin (1~100 ng/ml) for 60 min was not influenced compared to the corresponding control secretion (171 ± 17 ng, 0~4 min) (Fig. 2-lower). DMPP (10^{-4} M), a selective nicotinic receptor agonist in autonomic sympathetic ganglia, when perfused through the rat adrenal gland, evoked a sharp and rapid increase in CA secretion. As shown in Fig. 3-upper, DMPP- stimulated CA secretion following the loading with leptin (1~100 ng/ml) was greatly potentiated by 120% compared to the corresponding control secretion (299 ± 21 ng, 0~8 min), which was the peak release at 60~68 min. As illustrated in Fig. 3-lower, McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-receptor agonist (Hammer & Giachetti, 1982), perfused into an adrenal vein for 4 min caused an increased CA secretion to 151 ± 12 ng (0~4 min). However, in the presence of leptin (1~100 ng/ml), McN-A-343-evoked CA secretion was significantly increased by 133% of the corresponding control release (151 ± 12 ng/ 0~4 min), but at the low concentration of leptin it was not affected.

Effect of leptin on CA secretion evoked by Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

It has been found that Bay-K-8644 is a selective L-type calcium channel activator, which causes positive inotropy and vasoconstriction in isolated tissues and intact animals (Schramm et al, 1982; Wada et al, 1985), and enhances

basal Ca^{2+} uptake (Garcia et al, 1984) and CA release (Lim et al, 1992). Therefore, it was of interest to determine the effects of leptin on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. In the absence of leptin, Bay-K-8644 (10^{-5} M) given into the perfusion stream produced CA secretion of 128 ± 10 ng (0~4 min). However, in the presence of leptin (10 ng/ml), Bay-K-8644-stimulated CA secretion was significantly increased by 118% of the corresponding control secretion, but during the period of 0~19 min it was not affected, as shown in Fig. 4-upper.

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Geoger & Riley, 1989; Seidler et al, 1989). It may be extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic current regulated by intracellular calcium (Suzuki et al, 1992). When cyclopiazonic acid (10^{-5} M) was given into the perfusion stream, the CA secreted from the gland amounted to 139 ± 11 ng for 0~4 min. However, as shown in Fig. 4-lower, the pretreatment with leptin potentiated cyclopiazonic acid (10^{-5} M)-evoked CA secretion by 123% of the control response (100%).

Effect of the MAPK kinase inhibitor U0126 on leptin-induced potentiation of CA release evoked by ACh and DMPP from the perfused rat adrenal glands

An inhibitor of MAPK, U0126, is known to nullify the stimulatory effect of leptin on the synthesis of ^{14}C-catecholamines in cultured bovine adrenal medullary cells (Utsunomiya et al, 2001). Therefore, it was of interest to examine whether the MAPK inhibitor U0126 affects the stimulatory effect of leptin on ACh- and DMPP-evoked CA release in the perfused rat adrenal medulla. As shown in Fig. 5, U0126 (1 μm) completely abolished leptin (100

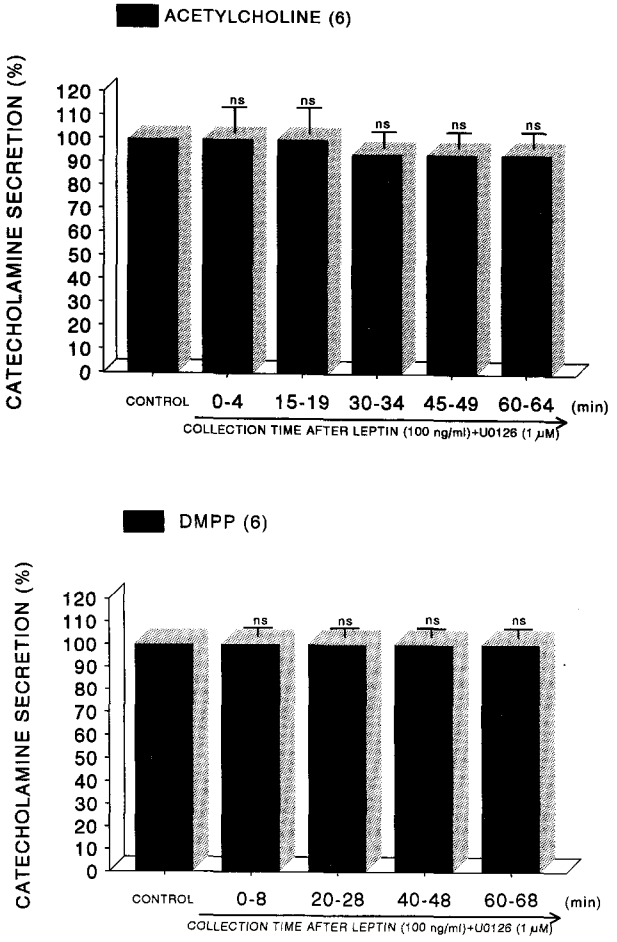

Fig. 5. Effects of leptin plus U0126 on catecholamine release evoked by acetylcholine (upper) and DMPP (lower) from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) or perfusion of DMPP (10^{-4} M) for 2 min was compared between group after preloading with 100 ng/ml leptin only (CONTROL) and group treated with 100 ng/ml leptin +1 M U0126 for 60 min, respectively. Ordinate: the amounts of CA secreted from the adrenal gland (%). Abscissa: Collection time of perfusate (min). ns: Statistically not significant.

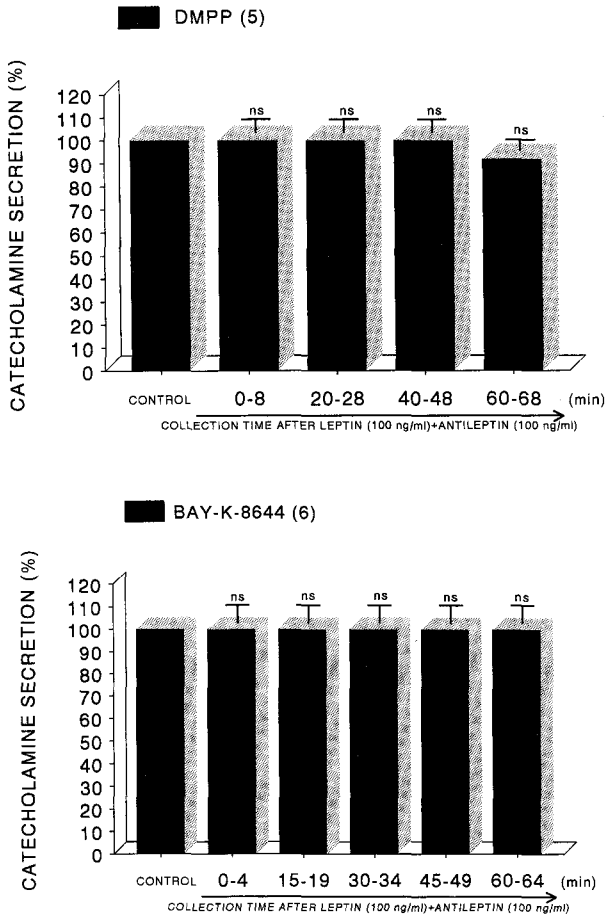

Fig. 6. Effects of leptin plus antileptin on catecholamine release evoked by DMPP (upper) and Bay-K-8644 (lower) from the isolated perfused rat adrenal glands. CA secretion by the perfusion of DMPP (10^{-4} M) for 2 min or Bay-K-8644 (10^{-5} M) for 4 min was compared between group after preloading with 100 ng/ml leptin only (CONTROL) and group treated with 100 ng/ml leptin +100 ng/ml antileptin for 60 min, respectively. Ordinate: the amounts of CA secreted from the adrenal gland (%). Abscissa: Collection time of perfusate (min). ns: Statistically not significant.

ng/ml)-induced enhancement of the CA secretory responses evoked by ACh and DMPP in comparison to the corresponding control response (100%).

Effect of antileptin on leptin-induced potentiation of CA release evoked by, DMPP and BAY-K-8644 from the perfused rat adrenal glands

It has also been found that, in this study, leptin enhanced the CA secretory response evoked by cholinergic stimulation in the perfused rat adrenal gland. Therefore, to study the relationship between leptin effect and leptin receptors, the effect of antileptin on CA secretory responses evoked by cholinergic nicotinic receptor-stimulation was examined. In the present study, as shown in Fig. 6-upper, DMPP-evoked CA release prior to the perfusion with leptin plus antileptin was 320±20 ng (0~8 min). The simultaneous perfusion of leptin (100 ng/ml) and antileptin (10 ng/ml) for 60 min no longer enhanced DMPP-evoked CA release in 5 experiments. As shown in Fig. 6-lower, the simultaneous perfusion of leptin (100 ng/ml) and antileptin (10 ng/ml) for 60 min completely abolished leptin-induced potentiation of the CA secretory responses evoked by Bay-K-644 and cyclopiazonic acid in comparison to their corresponding control responses (139±11 ng/ 0~4 min and 130±10 ng/ 0~4 min).

DISCUSSION

These experimental results demonstrate that leptin enhances the CA secretion evoked by cholinergic stimulation (both nicotininc and muscarinic receptors), but not that by membrane depolarization, through the activation of leptin receptors in the rat adrenal medulla. It seems that this facilitatory effect of leptin may be exerted by activation of U0126-sensitive MAPK-sensitive protein kinase through the leptin receptors, which is probably relevant to the activation of the dihydropyridine L-type Ca^{2+} channels located on the rat adrenomedullary chromaffin cells.

Based on the results that leptin enhanced the secretory responses of CA evoked by ACh, DMPP, McN-A-343, BAY-K-8644 and cyclopiazonic acid in the present study, the effectiveness of leptin in stimulating the release of endogenous CA, although it was very weak in the rat adrenal gland, may be due to the simultaneous actions of several intracellular mechanisms provoking transmembraneous Ca^{2+} influx, which acts as the most important mediator exocytosis (Kim & Westhead 1989). It has been found that the adrenal medulla possesses characteristics of postganglionic sympathetic neurons, and both L- and N-type voltage-dependent Ca^{2+} channels (VDCCs) have been identified in medullary chromaffin cells (Gandia et al, 1995). Adrenal CA secretion has been found to be mediated by muscarinic receptors as well as nicotinic receptors in various species (Harish et al, 1987; Nakazato et al, 1988), including the dog (Kimura et al, 1992). However, little is known about the involvement of VDCCs in contributing to the muscarinic receptor-mediated CA secretion (Uceda et al, 1994). It has been previously reported that leptin directly stimulates CA release from cultured porcine adrenal chromaffin cells in a manner highly dependent on extracellular Ca^{2+} (Takekoshi et al, 1999). Takekoshi and his colleagues (2001) further confirmed that leptin caused an increase of $[Ca^{2+}]_i$ in an extracellular Ca^{2+}-dependent manner, confirming that leptin-induced CA secretion is indeed dependent on external Ca^{2+}. Moreover, leptin-induced $[Ca^{2+}]_i$ and CA secretion was markedly suppressed by nicardipine and ω-conotoxin GVIA, indicating that VDCC, presumably both L-type and N-type Ca^{2+} channels, are involved in the mechanisms of leptin-induced CA secretion. Although there was no evidence that the increases of IP_3 are associated with increased cytosolic Ca^{2+} in this experimental system, they showed that leptin induced the mobilization of Ca^{2+} from intracellular storage sites, accompanied by increases of inositol IP_3, a messenger for mobilization of Ca^{2+} from intracellular storage sites. These findings indicate that leptin causes a sustained increase of $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from both extra- and intracellular pools. However, leptin (100 nM) caused little CA secretion in the absence of extracellular Ca^{2+} as previously reported (Takekoshi et al, 1999). Consistent with this, in the previous finding, it has been shown that xestospongin C (Xe C: a membrane-permeable blocker of IP_3-mediated Ca^{2+} release) (Gafni et al, 1997; Mattson et al, 2000) did not affect CA release caused by leptin. Kim and Westhead (1989) have demonstrated that Ca^{2+} entering across the plasma membrane was much more effective at triggering exocytosis than the Ca^{2+} released from internal stores. This led them to claim that Ca^{2+} released from intracellular storage sites does not induce exocytosis. Thus, it is possible that mobilization of Ca^{2+} from extracellular but not intracellular pools may contribute to CA secretion induced by leptin.

Besides the predominant role of Ca^{2+} influx for stimulating secretion processes, a norepinephrine release independent of extracellular Ca^{2+} has been observed in PC12 cells involving activation of protein kinase C (Pozzan et al, 1984). In the present investigation, the results that leptin enhanced CA secretion evoked by stimulation of muscarinic receptors with McN-A-343, a selective muscarinic M_1-receptor agonist, suggest that Ca^{2+} mobilization from intracellular store by the activation of muscarinic M_1-receptors might be involved in the leptin-induced enhancement of the CA secretory response in the rat adrenal medulla. In support of this hypothesis, the muscarinic receptor-mediated secretion of adrenal CA has been thought to be caused by Ca^{2+} mobilized from intracellular storage sites (Cheek & Burgoyne, 1987; Nakazato et al, 1988; Misbahuddin & Oka, 1988; Yamada et al, 1988). Furthermore, it has been shown that muscarinic stimulation generates a depolarizing signal, which triggers the firing of action potentials, resulting in the increased CA release in the rat chromaffin cells (Akaike et al, 1990), guinea pigs (Inoue & Kuriyama, 1991) and the perfused rat adrenal gland (Lim & Hwang, 1991). These observations are in line with a previous report showing that Bay-K-8644 almost trippled the peak secretory response to muscarine in perfused cat adrenal glands (Ladona et al, 1987; Uceda et al, 1994). In the present experiment, leptin also potentiated the CA secretion induced by Bay-K-8644, which is found to evoke the release of CA by increasing Ca^{2+} influx through L-type Ca^{2+} channels in chromaffin cells (Garcia et al, 1984). These findings that leptin potentiated CA secretion evoked by Bay-K-8644, not by high K^+, suggest that leptin activates directly the voltage-dependent Ca^{2+} channels. In the bovine chromaffin cells, stimulation of nicotinic, but not muscarinic ACh receptors is known to cause CA secretion by increasing Ca^{2+} influx largely through voltage-dependent Ca^{2+} channels (Oka et al, 1979; Burgoyne, 1984). In the light of this notion, the present finding that leptin

enhances DMPP-evoked CA secretion is thought to be due to the increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors.

It is felt that the facilitatory effect of leptin on CA secretory responses evoked by cholinergic stimulation may be associated with the mobilization of intracellular Ca^{2+} from the cytoplasmic calcium store. This indicates that leptin increases the release of Ca^{2+} from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the secretion of CA. It has been shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (Ilno, 1989) is almost completely abolished by treatment with cyclopiazonic acid during the proceeding Ca^{2+} load (Suzuki et al, 1992). This is consistent with the findings obtained in skinned smooth muscle fibers of the longitudinal layer of the guinea-pig ileum, where Ca^{2+}-uptake was also inhibited by cylopiazonic acid (Uyama et al, 1992). Suzuki and his coworkers (1992) have shown that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increase in the subsequent Ca^{2+} release from those storage sites. Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide metabolism, resulting in the formation of inositol 1,4,5-trisphosphate, which induces the mobilization of Ca^{2+} from the intracellular pools (Cheek et al, 1989; Challis et al, 1991). Furthermore, intracellular events such as increased generation of the second messenger IP_3 form intracellular Ca^{2+} stores, which are both provoked by leptin in cultured porcine chromaffin cells (Takekoshi et al, 2001) have been already found. In the light of this finding, it is plausible that leptin enhances the CA secretory responses evoked by cholinergic stimulation through the mobilization of the intracellular Ca^{2+}. However, in the present study, it is uncertain whether the stimulatory effect of leptin on Ca^{2+} movement from intracellular pools is due to its direct effect on the PI response or the indirect effects.

Despite the responsiveness of the rat adrenal medulla to leptin, the leptin-receptors involved in mediating CA secretion have not yet been characterized. Leptin receptors exist basically in two forms, and differ only in the length of their intracellular domains. The long form of the leptin receptors (ObRb) is expressed at high levels in the hypothalamus, which is presumed to be major site of action for leptin. Leptin receptors are believed to function as activators of a janus kinase through signaling transductions and activators of transcription (JAK-STAT) pathways (Tartaglia et al, 1995; Schwartz et al, 1996). The short form of the leptin receptors (ObRa) is present in a number of peripheral organs such as the lung, liver, and the pancreas. Indeed, growing evidence indicates that leptin has significant direct effects on peripheral tissues, including pancreatic R cells and the adrenal cortex (Leclercq-Meyer et al, 1996; Bornstein et al, 1997; Emilsson et al, 1997; Tanizawa et al, 1997; Pralong et al, 1998).

Cao & his colleagues (1997) demonstrated the presence of leptin receptors (Ob-Ra) on epinephrine-secreting chromaffin cells in rat adrenal medulla, suggesting that leptin may directly affect the adrenal medulla. On the other hand, it has been shown that long form of leptin receptors (Ob-Rb), were highly expressed in the mouse and human adrenal medulla, respectively (Hoggard et al, 1997; Glasow et al, 1998).

Based on these results, in the present study, leptin-induced enhancement of CA secretion evoked by cholinergic nicotinic receptor stimulation as well as by Bay-K-8644 was blocked in the presence of antileptin. This result indicates that leptin enhances CA release through the activation of leptin receptors located on the rat adrenomedullary chromaffin cells. In support of this notion, it has been found that the expression of leptin receptor (Ob-Rb) mRNA in cultured porcine adrenal medullary cells was initially confirmed (Takekoshi et al, 1999). Yanagihara & his colleagues (2000) have also suggested that leptin stimulates CA synthesis through its receptors in bovine adrenal medullary cells.

In contrast, Bornstein & his colleagues (1997) reported that human leptin (6.25 nM) failed to stimulate CA release in human adrenal medullary cells, which does not agree with the results presented here. The reasons for this difference are unclear. They used whole adrenal tissue for cell culture instead of the perfused model of adrenal medulla as used in this study. Also, species difference (rat vs. human), difference of leptin used (murine vs. human), difference of leptin concentration used (1~100 ng/ml vs. 6.25 nM) should be considered.

Leptin is found to activate MAPK in a stable Chinese hamster ovary cell line expressing ObRa (Yamashita et al, 1998). It has also been shown that leptin activated MAPK in cultured adrenal medullary cells, and tyrosine hydroxylase can be phosphorylated and activated by MAPK (Haycock et al, 1992; Halloran & Vulliet 1994). Combining previous data and the present results implies that leptin phosphorylates and activates tyrosine hydroxylase through an ObR-MAPK pathway, resulting in the CA release. This hypothesis is further supported by the present finding that U0126, an inhibitor of MAPK kinase, abolished the stimulatory effect of leptin on ACh- or DMPP-evoked CA release. Thus, based on these results, it seems likely that leptin-induced potentiation of CA release is relevant to the activation of U0126-sensitive MAP kinase in the rat adrenomedullary cells.

Thus, it is still possible that the leptin-induced stimulatory effect of CA release may converge on the MAPK pathway. Further studies will be needed to clarify the interaction and/or relationship between the MAPK pathways induced by leptin in chromaffin cells.

In conclusion, these experimental results suggest that leptin enhances the CA secretion from the rat adrenal medulla evoked by cholinergic stimulation (both nicotininc and muscarinic receptors), but does not that by membrane depolarization. It seems that this facilitatory effect of leptin may be mediated by activation of U0126-sensitive MAPK-sensitive protein kinase through the leptin receptors, which is probably relevant to the activation of the dihydropyridine L-type Ca^{2+} channels located on the rat adrenomedullary chromaffin cells.

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