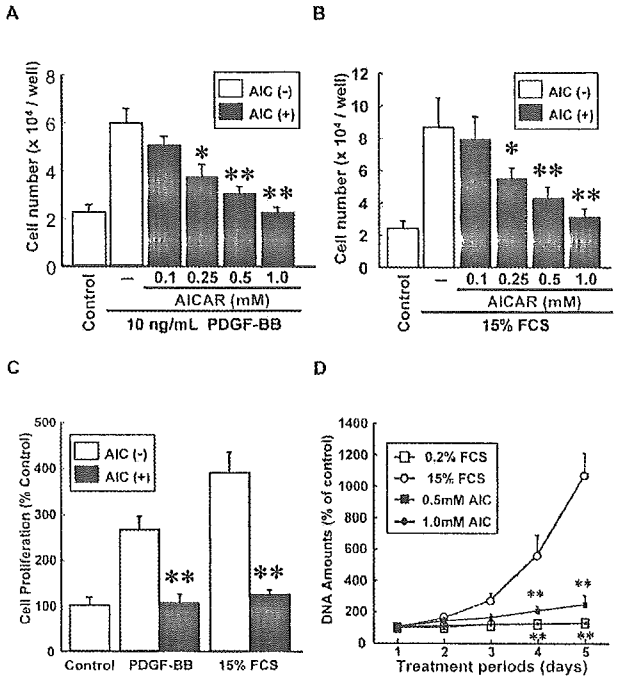

Figure 1. AICAR inhibits HASMC proliferation and DNA synthesis. A and B, Dose-dependent effect of AICAR on cell number of HASMCs stimulated by PDGF-BB (A) or FCS (B), respectively. Quiescent cells on 6-well dish were stimulated with PDGF-BB (10 ng/mL) or FCS (15%) in the presence (solid bars) or absence (open bars) of indicated concentrations of AICAR for 4 days. Cell number was determined by the cell counting. C, Effect of AICAR on Alamar Blue reduction. Quiescent HASMCs on 96-well plate were treated as described above. After 4 days, culture medium was replaced with DMEM containing 10% Alamar Blue. Cell proliferation was determined by measuring fluorescence intensity. Cell proliferation is expressed as a percentage of that in the nonstimulated condition (control). D, Effect of AICAR on DNA synthesis. Quiescent HASMCs on 96-well plate were treated as described above and harvested at the indicated time points. Total cellular DNA content was determined by measuring fluorescence intensity. DNA content is expressed as a percentage of that harvested at day 0 (just before mitotic stimulation). Data are shown as mean±SEM from at least 3 separate experiments. $^{*}P<0.05$, $^{**}P<0.01$ as compared with PDGF or 15% FCS alone.

(data not shown). Furthermore, treatment with 15% FCS increased DNA synthesis in HASMCs, and AICAR significantly suppressed the increase in DNA synthesis in a dose-dependent manner (Figure 1D). Notably, AICAR treatment did not reduce DNA amounts in cells compared with the control cells treated with 0.2% FCS, suggesting the inhibitory effect of AICAR on DNA synthesis rather than the loss of cellular DNA due to a cytotoxic effect.

AICAR Activates AMPK in HASMCs, RASMCs, and Isolated Aortic Strips

Next, we investigated the effect of AICAR on the phosphorylation of AMPK in HASMCs and RASMCs by Western blot analyses using an antibody specific for the Thr-172 phosphorylation of α-subunit of AMPK (α-AMPK). Treatment with AICAR for 2 hours markedly increased the phosphorylation of α-AMPK compared with the vehicle-treated control in HASMCs and RASMCs (Figure 2A and 2B). Although the

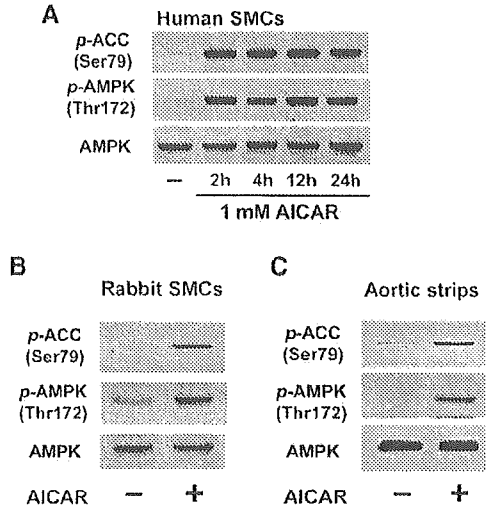

Figure 2. Effect of AICAR on the phosphorylation of AMPK and ACC. A and B, Quiescent HASMCs (A) or RASMCs (B) were treated without or with 1 mmol/L AICAR for indicated periods (A) or for 2 hours (B). C, Endothelium-denuded rabbit aortic strips were treated without or with 1 mmol/L AICAR for 4 hours. Western blot analyses were performed using 30 μg of protein in each lane. AMPK phosphorylation (*p*-AMPK) and ACC phosphorylation (*p*-ACC) was assessed. Total AMPK was detected with anti-pan-α-AMPK antibody. Representative blots of 3 independent experiments are shown.

increased Thr-172 phosphorylation of α-AMPK is indicative of the activation of this kinase, we also immunoblotted with anti-phospho-ACC (Ser-79) antibody to ascertain whether increased phosphorylation of AMPK had effects on downstream target proteins. In accordance with AMPK activation, phosphorylation of ACC was markedly elevated in AICAR-treated HASMCs and RASMCs (Figure 2A and 2B). We investigated the time course of AICAR effect on phosphorylation of AMPK and ACC. Phosphorylation of these molecules by AICAR was sustained over 24 hours in HASMCs (Figure 2A).

Further, we investigated whether AICAR could activate AMPK in isolated aortic strips. Endothelium-denuded aortic strips were stimulated with 1 mmol/L AICAR for 2 hours. Increased phosphorylation of AMPK and ACC were observed in AICAR-treated strips (Figure 2C). These results demonstrate that AICAR activates AMPK and regulates downstream enzyme ACC in primary cultured SMCs (in vitro) and in isolated aortic strips (ex vivo).

Inhibition of AMPK Activity by Inhibitors of AICAR Function or DN-AMPK Blocks the Growth-Suppressive Effect of AICAR

To exclude the possibility that the inhibitory effect of AICAR on SMC proliferation was caused by mechanisms other than AMPK activation, we investigated the effects of 2 different inhibitors of AICAR function, dipyridamole and AMDA. Dipyridamole inhibits transport of AICAR into cells by inhibiting an adenosine transporter, and AMDA inhibits the phosphorylation of AICAR by blocking the adenosine kinase in the cells.[19,29,30] Pretreatment with dipyridamole completely blocked the inhibitory effect of AICAR on proliferation

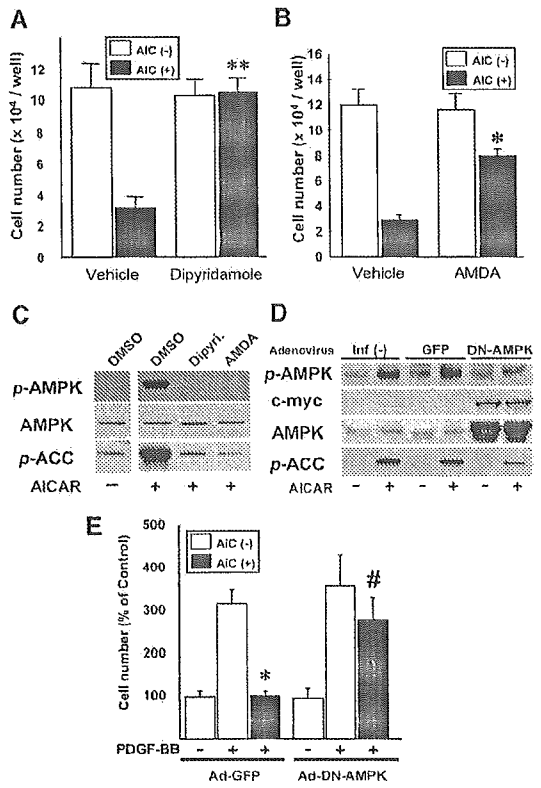

Figure 3. Effects of the inhibition of AMPK activity by inhibitors of AICAR function or by DN-AMPK on the antiproliferative effect of AICAR. A and B, Quiescent HASMCs were pretreated with dipyridamole (10 μmol/L; A), AMDA (10 μmol/L; B), or vehicle (DMSO) for 30 minutes. Cells were then incubated for 4 days without (open bars) or with (solid bars) 1 mmol/L AICAR in the presence of 15% FCS and inhibitors. Cell counting assay was performed. Data are mean±SEM (n=6) from 3 independent experiments. The significance versus AICAR-treated cells with vehicle was expressed as $^*P<0.01$. C, Quiescent HASMCs were pretreated with inhibitors or vehicle (DMSO) for 30 minutes and then stimulated without or with 1 mmol/L AICAR for 2 hours. D, Quiescent HASMCs without infection [Inf (-)] or infected with the indicated adenoviral vector were treated without or with 1 mmol/L AICAR for 2 hours. Western blot analyses were performed. Representative blots of 3 independent experiments are shown. E, Quiescent HASMCs infected with the indicated adenoviral vector were incubated for 4 days without (open bars) or with (solid bars) 1 mmol/L AICAR in the presence of 15% FCS. Cell counting assay was performed. $^*P<0.01$ as compared with 15% FCS alone in Ad-GFP-infected cells; $\#P<0.01$ as compared with AICAR-treated Ad-GFP-infected cells.

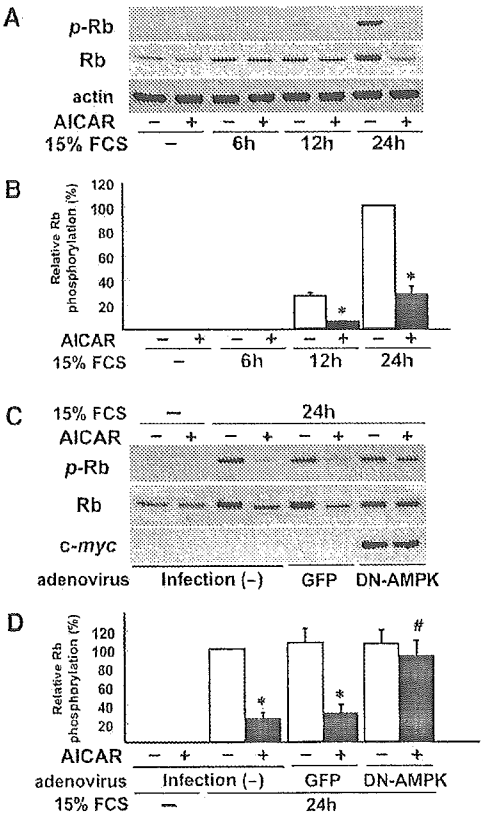

Figure 4. Effect of AICAR on Rb phosphorylation in HASMCs. A and B, Quiescent HASMCs were pretreated without or with 1 mmol/L AICAR in DMEM containing 0.2% FCS for 4 hours. Then cells were stimulated without or with 15% FCS for the indicated periods. C and D, HASMCs were not infected [Infection (-)prsqb] or infected with Ad-GFP or Ad-DN-AMPK and were made quiescent. Cells were then treated without or with 1 mmol/L AICAR for 4 hours and stimulated with 15% FCS for 24 hours. Western blot analyses were performed to detect total (Rb), phosphorylated Rb (*p*-Rb), and actin to confirm equal loading. The expression of DN-AMPK was confirmed by blotting with anti–c-*myc* antibody. Representative blots (A and C) and relative levels of Rb phosphorylation (B and D) are shown. The data are quantified from 3 independent experiments. $^*P<0.01$ as compared with 15% FCS alone; $\#P<0.01$ as compared with AICAR-treated Ad-GFP-infected cells.

(Figure 3A). AMDA partially but significantly blocked the inhibitory effect of AICAR on proliferation (Figure 3B). Pretreatment with these inhibitors completely inhibited AICAR-induced phosphorylation of AMPK and ACC (Figure 3C). These results indicated that ZMP formation through both transport and phosphorylation of AICAR is required for the suppression of growth by AICAR, suggesting that AMPK activation is a key process for an inhibitory effect of AICAR on SMC proliferation.

To further confirm the involvement of AMPK on growth-suppressive effect of AICAR, we performed the experiments using an adenoviral vector expressing DN-AMPK, which has been reported to inhibit AMPK activation as a nonphosphorylatable T172A mutant.[25–26] Overexpression of DN-AMPK, but not of green fluorescent protein (GFP) (control), suppressed AICAR-induced phosphorylation of AMPK and ACC (Figure 3D). Overexpression of DN-AMPK was confirmed by Western blotting using both anti–c-*myc* and anti–pan-α-AMPK antibodies. In HASMCs infected with Ad-GFP, AICAR completely suppressed proliferation (Figure 3E) as observed in Figure 1A. DN-AMPK significantly inhibited the suppressive effect of AICAR on proliferation. These results indicate the involvement of AMPK on AICAR-induced suppression of SMC proliferation.

AICAR Inhibits Phosphorylation of Rb

Next, we examined the effect of AICAR on the phosphorylation of Rb stimulated with 15% FCS or PDGF, as phosphorylation of Rb has been reported to be a critical and common event during cell proliferation process.[7–9,31] Increased phosphorylation of Rb was detected 12 hours after stimulation with FCS. Rb phosphorylation was further in-

creased in a time-dependent manner, indicating the cell cycle progression induced by FCS. AICAR significantly inhibited FCS-induced Rb phosphorylation (Figure 4A and 4B). AICAR also strongly suppressed PDGF-induced Rb phosphorylation (supplemental Figure S2A). These results suggest that AICAR suppresses a G1 event in cell cycle progression. This suppressive effect of AICAR on Rb phosphorylation was inhibited by overexpression of DN-AMPK but not by that of the control GFP (Figure 4C and 4D and supplemental Figure S2B).

AMPK Induces G1 Cell Cycle Arrest but Not Apoptosis

Reduction in cell number induced by AMPK activation could be the result of the inhibition of proliferation or increased cell death. To distinguish these possibilities, we first investigated the effect of AMPK on cell cycle progression using a flow cytometry analysis. Compared with control cells treated with 15% FCS, AICAR significantly increased the cells in the G0/G1 phase (from 76.0±2.2% to 88.6±1.9%) and decreased those in S (from 14.6±0.8% to 5.7±1.4%) and G2/M phase (from 9.4±1.5% to 5.7±1.5%) (Figure 5A). This effect of AICAR was statistically significant ($P<0.01$, n=5) and was almost completely inhibited either by coincubation with dipyridamole or by overexpression of DN-AMPK (supplemental Figure S3). These data suggest AMPK activation causes G1 arrest in HASMCs.

To examine the second possibility, we investigated whether AICAR could induce cell death. In trypan blue exclusion assay, DEM significantly decreased viable cell number. In contrast, no difference was observed in the rate of appearance for dead cells between AICAR-treated and vehicle-treated HASMCs (Figure 5B). Using a cell death ELISA quantitative assay, no differences were observed in the rates of cytoplasmic DNA-histone complex formation between HASMCs treated with AICAR and those with 15% FCS alone (Figure 5C). In addition, increased population in sub-G1 was not observed even after 72 hours in AICAR-treated HASMCs (data not shown). These data indicate that AMPK-induced cell number reduction in HASMCs is due to the inhibition of cell proliferation rather than cell death.

AMPK Increases the Expression of $p21^{CIP}$

We further investigated the effect of AMPK on the protein expression of CDKIs $p21^{CIP}$ and $p27^{KIP}$. Increased expression of $p21^{CIP}$ protein but not of $p27^{KIP}$ was observed in AICAR-treated HASMCs compared with those treated with 15% FCS alone from 6 hours to 24 hours after stimulation with FCS (Figure 6A and 6B). FCS stimulation decreased $p27^{KIP}$ expression. AICAR did not block the FCS-induced reduction of $p27^{KIP}$ (Figure 6A, middle panel). Expression of $p21^{CIP}$ has been reported to be regulated both in p53-dependent and -independent manners. To test whether AMPK increases the $p21^{CIP}$ expression through the activation of p53, we examined the effect of AICAR on expression and Ser-15 phosphorylation of p53. AICAR increased phosphorylated p53 and its protein expression, and also increased the expression of $p21^{CIP}$ (Figure 6C) in a dose-dependent manner. The increase in p53 protein was associated with an increased $p21^{CIP}$ level.

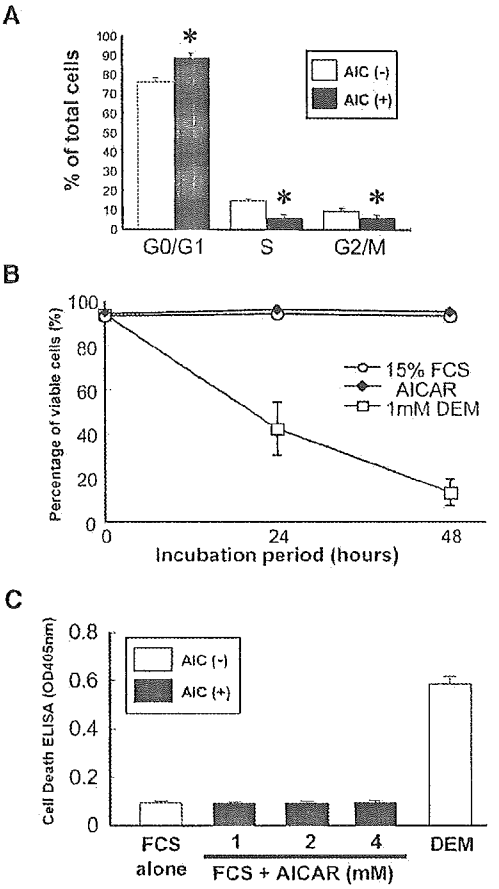

Figure 5. Effect of AICAR on cell cycle progression, viability, and apoptosis. A, Cell cycle distribution in AICAR-treated HASMCs. Data are expressed as a percentage of total cells. Each value represents mean±SEM from 5 independent experiments. *$P<0.01$ as compared with 15% FCS alone (open bars). B, Viability of HASMCs treated with AICAR. HASMCs were treated without (open circles) or with (closed squares) 1 mmol/L AICAR in DMEM containing 15% FCS. As a positive cytotoxic control, treatment with 1 mmol/L DEM was performed in DMEM containing 15% FCS. After the indicated period, viability was determined by trypan blue exclusion assay. Results are expressed as a percentage of viable cells to total cells counted. C, AICAR does not induce apoptosis in HASMCs. HASMCs were treated without (open bars) or with (solid bars) indicated concentrations of AICAR or 1 mmol/L DEM in DMEM containing 15% FCS for 24 hours. Apoptosis was assessed using the cell death ELISA and expressed as OD at 405 nm. Results (mean±SEM) were from 3 independent experiments.

We further investigated the effect of AICAR on the mRNA expression of $p21^{CIP}$ and p53 using a real-time RT-PCR analysis. AICAR increased the expression of $p21^{CIP}$ mRNA, whereas no significant change was observed in the mRNA expression of p53 (supplemental Figure S4A), indicating the transcriptional and post-transcriptional mechanisms for $p21^{CIP}$ and p53 upregulation, respectively. We further investigated whether p53 is functionally activated in HASMCs treated with AICAR using a reporter assay system. This dual-luciferase assay revealed that p53-dependent transcription in AICAR-treated cells significantly increased compared with both control cells and those treated with 15% FCS alone (supplemental Figure S4B).

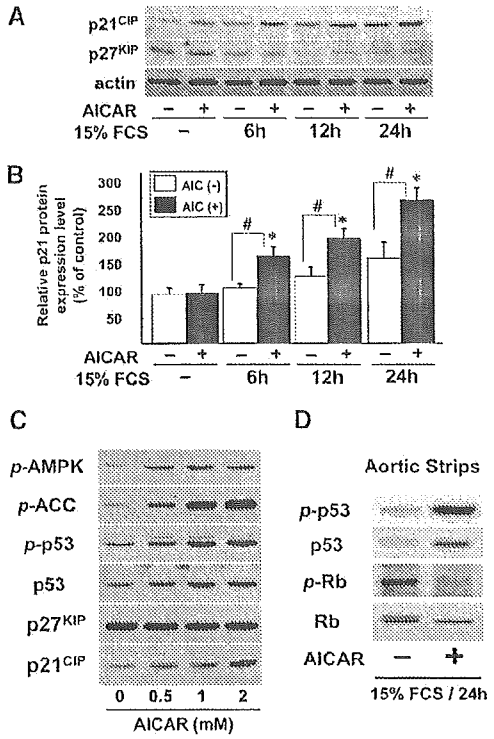

Figure 6. Effect of AICAR on the expression of p21, p27, and p53. A and B, Quiescent HASMCs were treated without (open bars) or with (solid bars) 1 mmol/L AICAR for the indicated periods in DMEM containing 15% FCS. Controls without FCS stimulation were harvested just before adding FCS (lane 1) or just after adding AICAR alone (lane 2). Representative blots (A) for $p21^{CIP}$ and $p27^{KIP}$ protein from 3 independent experiments and quantitative data for $p21^{CIP}$ protein (B) are shown. Actin was detected to confirm equal loading. *$P<0.05$ as compared with control incubated without 15% FCS nor AICAR; #$P<0.05$ as compared with 15% FCS alone at each time point. C, Quiescent HASMCs were treated without or with the indicated concentrations of AICAR in DMEM without FCS for 3 hours. D, Endothelium-denuded aortic strips were treated without or with 1 mmol/L AICAR for 4 hours, and then FCS was added at the final concentration of 15%. After 24 hours, strips were harvested. Representative blots of 3 independent experiments are shown.

Finally, we investigated whether AMPK activation could exhibit several effects in isolated rabbit aortas, as observed in cultured SMCs. As observed in Figure 2C, AICAR significantly increased the phosphorylation of AMPK and ACC in our ex vivo experimental system. Furthermore, increased p53 phosphorylation and p53 protein expression were accompanied with the decreased Rb phosphorylation in AICAR-treated aortic strips (Figure 6D). The increased levels of phosphorylated p53 and p53 protein were dependent on the activation of AMPK, as either dipyridamole or AMDA completely blocked these AICAR-induced changes in p53 (supplemental Figure S4C). These findings indicate that AMPK exhibits the growth-inhibitory effect in aortic SMCs in vivo, as observed in cultured SMCs and in aortic strips.

Discussion

In the present study, we have demonstrated for the first time that AMPK inhibited FCS- and PDGF-induced proliferation in human aortic SMCs. Similar results were also obtained in rabbit aortic SMCs. The mechanism of growth suppression induced by AMPK turned out to be a cell cycle arrest at G1 phase but not by an apoptosis. We further investigated how AMPK induces cell cycle arrest in SMCs, and found that activation of AMPK increased the CDKI $p21^{CIP}$ protein through the upregulation of p53, which in turn inhibited the Rb phosphorylation required for cell cycle progression.

Transport of AICAR into cells has been studied previously.[20,29,32] Lopez et al reported that adenosine inhibited the accumulation of ZMP in AICAR-treated Jurkat cells via competition with AICAR transport into the cells.[32] Dipyridamole and AMDA were reported to block adenosine-induced apoptosis by inhibiting AMPK activation.[29] Further, adenosine and 5-iodotubercidine, another adenosine kinase inhibitor, were reported to inhibit apoptosis and AMPK phosphorylation in B-cell chronic lymphocytic leukemia cells treated with AICAR.[20] Because of these reports, we investigated the effect of dipyridamole and AMDA on AICAR-induced growth suppression. As expected, both inhibitors blocked the inhibitory effect of AICAR on SMC proliferation, as well as AICAR-induced phosphorylation of AMPK and ACC. These observations indicate that both uptake and phosphorylation of AICAR are necessary for the activation of AMPK by AICAR and AICAR-induced suppression of SMC proliferation, suggesting that AMPK activation is required for the growth suppression of AICAR. Adenosine was reported to induce apoptosis in HASMCs via A2b adenosine receptor and cAMP-dependent pathways.[33] Although AICAR is an adenosine analogue, treatment of HASMCs with adenosine or AICAR seems to induce totally different cellular events, apoptosis or growth-suppression without apoptosis, respectively. We also demonstrated that DN-AMPK inhibited the growth suppression by AICAR, which further supports the involvement of AMPK in AICAR-induced suppression of SMC proliferation.

Recently, Nagata et al reported that AICAR-induced AMPK activation inhibited Ang II-induced proliferation of VSMCs derived from rat aortas.[22] Their observations are consistent with our present study, although the mitotic stimulations used were different. To confirm their observations, we performed several preliminary experiments. Indeed, AICAR inhibited proliferation induced by Ang II in HASMCs, and we also found that AICAR induced cell cycle arrest in Ang II-treated HASMCs (unpublished data, 2004). These findings indicate activation of AMPK by AICAR induces cell cycle arrest and suppresses proliferation in SMCs treated with either PDGF-BB, Ang II, or FCS.

As mentioned above, AICAR has been reported to be able to either induce or inhibit apoptosis depending on the cell types.[17–20] In HASMCs, AICAR did not induce apoptosis. AICAR may have an anti-apoptotic effect in HASMCs, as AICAR treatment significantly reduced sub-G1 fraction in SMCs growing without rendering quiescent (10.6±2.2% of control cells or 4.7±0.9% of those treated with AICAR were located in sub-G1 fraction, respectively; $P<0.01$, n=5 each; unpublished data, 2004). Further intensive investigations are required to elucidate for the anti-apoptotic effect of AICAR in SMCs.

In the present study, AICAR increased p53 and p21 protein expression, as well as Ser-15 phosphorylated p53, in HASMCs and isolated rabbit aortas. Our results are consistent with a report by Imamura et al.[34] They reported that AICAR suppressed proliferation via p53 phosphorylation and its accumulation in HepG2 cells. It has also been reported that Ser-15 modification of p53 results in decreased binding affinity between mdm2 and p53, thereby suppressing the degradation of p53 protein, resulting in p53 accumulation.[35] In response to stresses such as hypoxia or DNA damage, p53 is activated by several mechanisms, including the phosphorylation, and the activated p53 induces either cell cycle arrest or apoptosis.[36] In our present study, activation of p53 signaling in HASMCs by AICAR was confirmed using both reporter-gene analysis and RT-PCR analysis for $p21^{CIP}$.

It has been reported that adenovirus-mediated overexpression of p21 or p53 inhibits vascular SMC proliferation and suppresses neointima formation in the rat carotid artery,[37,38] suggesting that p21 or p53 functions to suppress SMC proliferation and neointima formation.

The most striking effect of AICAR was the induction of a CDKI $p21^{CIP}$. Inactivation of CDKs by CDKIs maintains Rb at hypophosphorylated state, which keeps E2F inactive.[39] E2F is required for induction of several factors essential for S phase progression; thus, maintaining E2F in an inactive state leads to G1 arrest. In our present study, upregulation of $p21^{CIP}$ and decline in Rb phosphorylation by AICAR were observed, indicating that $p21^{CIP}$ plays a major role in AICAR-induced G1 arrest in HASMCs.

In the present study, we have demonstrated that AICAR treatment increased Ser-15 phosphorylation and protein expression of p53 in HASMCs and isolated rabbit aortas. Because either dipyridamole or overexpression of DN-AMPK completely blocked these events, it is suggested that either AMPK itself or downstream kinase is involved in AICAR-induced p53 upregulation. Although we have not tested whether Ser-15 phosphorylation of p53 is necessary for the AICAR-induced cell cycle arrest, during the preparation of this article, Jones et al[40] reported that activation of AMPK either by glucose limitation, treatment with AICAR, or overexpression of constitutive active AMPK induced a G1 cell cycle arrest via AMPK-dependent Ser-15 phosphorylation of p53 in primary mouse embryonic fibroblasts. In addition, they demonstrated that Ser-15 phosphorylation of p53 was required for AMPK-induced cell cycle arrest using mouse embryonic fibroblasts derived from $p53^{Ser18Ala}$ mice, in which Ser-18 of mouse p53 corresponding to Ser-15 of human p53 was mutated. Therefore, we speculated that AICAR phosphorylated p53 protein and subsequently increased the amount of both p53 protein and phosphorylated p53 protein because the phosphorylation of p53 protein has been reported to increase the stability of this protein.[35,36]

The Ser-15 phosphorylation of p53 has been reported to be mediated by 3 distinct protein kinases, Ataxia-Telangiectasia Mutated (ATM), ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) in response to DNA damage.[10–11,41] Jones et al[40] demonstrated that AICAR-induced Ser-15 phosphorylation of p53 was ATM-independent but AMPK-dependent, suggesting the possibility that AMPK may directly phosphorylate Ser-15 of p53. In our present study, it was not tested whether AMPK activation by AICAR could activate ATM, ATR, or DNA-PK and whether these kinases could phosphorylate p53 under our experimental conditions. Further investigation is required to understand the mechanisms of AICAR-induced p53 phosphorylation and accumulation in HASMCs.

In conclusion, this is the first study to show that activation of AMPK by AICAR effectively suppressed cell cycle progression in primary human VSMCs and isolated rabbit aortas, suggesting that AMPK could be a target for the prevention of vascular proliferative disorders such as atherosclerosis.

Acknowledgments

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A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice

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Abstract

Brain serotonin (5-hydroxytryptamine; 5-HT) systems contribute to regulate eating behavior and energy homeostasis. 5-HT2C receptors and 5-HT1B receptors have been shown to mediate anorexic effects of 5-HT drugs such as D-fenfluramine, which stimulates 5-HT release and inhibits 5-HT reuptake, and *m*-chlorophenylpiperazine (mCPP), a 5-HT2C receptor agonist. Here, we report that 24-h fasting increased the expression of hypothalamic 5-HT2C receptor and 5-HT1B receptor genes in association with increases in plasma active ghrelin levels compared with fed state in mice. Treatment with mCPP or fenfluramine significantly inhibited the increases in plasma active ghrelin levels. mCPP or fenfluramine significantly increased the expression of hypothalamic pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript genes while having no significant effects on the expression of hypothalamic neuropeptide Y, agouti-related protein, and ghrelin genes. These results suggest that there is a negative feedback system between brain 5-HT systems and plasma active ghrelin levels in energy homeostasis in mice.

Keywords: Serotonin; 5-HT2C receptor; 5-HT1B receptor; Ghrelin; Neuropeptide; POMC; CART; AGRP; Hypothalamus; Eating behavior; Energy homeostasis; Gene expression; mCPP; Fenfluramine

Mice with a mutated serotonin (5-hydroxytryptamine; 5-HT) 5-HT2C receptor gene display leptin-independent hyperphagia and hyperactivity that leads to a late onset of obesity associated with the secondary leptin resistance [1–3]. Several lines of evidence indicate that 5-HT2C receptors contribute to mediate the anorexic actions of 5-HT agonists such as *m*-chlorophenylpiperazine (mCPP) and 5-HT reuptake inhibitors such as D-fenfluramine and D-norfenfluramine [1,4,5]. These finding indicate that brain 5-HT systems via 5-HT2C receptors contribute to the regulation of eating behavior and energy homeostasis. In addition, 5-HT1B receptors have been suggested to mediate the anorexic actions of mCPP and D-fenfluramine in mice [6]. Activation of 5-HT2C receptor and 5-HT1B receptor signaling might therefore coordinate to suppress food intake.

The link between brain 5-HT systems and neuropeptides or gut peptides in the regulation of feeding and energy homeostasis, however, is still unclear. Ghrelin is an orexigenic peptide, secreted by stomach [7–9]. Fasting elevates plasma ghrelin levels, while feeding reduces the levels in animals and humans [8–10]. There are two forms of ghrelin, an active ghrelin with bioactivity and des-acyl ghrelin without biologic activity [10–12]. Because only the acylated form of ghrelin is active, measuring the acylated form of ghrelin might be advantageous.

In the present study, to determine the relationship between brain 5-HT systems and plasma ghrelin levels in the regulation of energy homeostasis, we investigated: (1) alterations of the expression of hypothalamic 5-HT2C and 5-HT1B receptor genes induced by fasting and feeding, (2) the effects of mCPP or fenfluramine on plasma active ghrelin levels, and (3) the effects of mCPP or fenfluramine on the gene expression of hypothalamic neuropeptides that

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are involved in regulating feeding behavior and energy homeostasis in mice.

Materials and methods

Animals and drug treatment. Four-week-old male C57BL/6J mice were purchased from Japan CLEA. Mice were individually housed in cages with free access to water and chow pellets in a light- (12 h on/12 h off; lights on at 08:00 h and lights off at 20:00 h) and temperature (20–22 °C)-controlled environment. Animals were acclimatized to the laboratory environment for 1 week before the experiment. mCPP and fenfluramine were purchased from Sigma Chemical (St. Louis, MO, USA). mCPP (5 mg/kg) and fenfluramine (3 mg/kg) were dissolved in saline. Drugs were administered at 10 a.m. to12 p.m. mCPP and fenfluramine were purchased from Sigma Chemical, Japan. mCPP (5 mg/kg) and fenfluramine (3 mg/kg) were dissolved in saline. The doses of mCPP (5 mg/kg) and fenfluramine (3 mg/kg) were set on the evidence that either mCPP or fenfluramine-induced hypophagia is attenuated in 5-HT2C receptor-deficient mice [1,4].

In the first experiment, 5-week-old male C57BL/6J mice in either a fed or 24-h fasted state were decapitated and blood was collected. Whole blood was mixed with EDTA-2Na (2 mg/ml) and aprotinin (500 kIU/ml) to determine the plasma active ghrelin levels. The hypothalamus was removed for RNA extraction.

In the second experiment, 5-week-old male C57BL/6J mice were deprived of food for 23 h. The following morning, the animals were intraperitoneally injected with saline or mCPP (5 mg/kg) or fenfluramine (3 mg/kg). Sixty minutes later, the animals were decapitated and blood was drawn; the animals were not fed. The hypothalamus was removed for RNA extraction.

The animal studies were conducted under protocols in accordance with the Institutional Guidelines for Animal Experiments at Tohoku University.

Real-time quantitative RT-PCR. Total RNA was isolated from mouse hypothalamic tissue using the RNeasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's directions, and cDNA synthesis was performed using a Super Script III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Rockville, MD) using 1 μg total RNA. cDNA synthesized from total RNA was evaluated in a real-time polymerase chain reaction (PCR) quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Mannheim, Germany). The primers used were as follows. For mouse 5-HT1B receptor, sense, 5′-TGC CTG CTG GTT TCA CAT-3′, 5′-ATA GAT GTG TGG AGC TGG TG-3′, antisense, 5′-GCG CAC TTA AAG CGT ATC A-3′; 5-HT2C receptor, sense, 5′-CTG AGG GAC GAA AGC AAA G-3′, antisense, 5′-CAC ATA GCC AAT CCA AAC AAA C-3′; pro-opiomelanocortin (POMC), sense, 5′-ATA GAT GTG TGG AGC TGG TG-3′, antisense, 5′-GGC TGT TCA TCT CCG TTG-3′; for mouse cocaine- and amphetamine-regulated transcript (CART), sense, 5′-CTG GAC ATC TAC TCT GCC GTG G-3′, antisense, 5′-GTT CCT CGG GGA CAG TCA CAC AGC-3′; for mouse neuropeptide Y (NPY), sense, 5′-GCT TGA AGA CCC TTC CAT TGG TG-3′, antisense, 5′-GGC GGA GTC CAG CCT AGT GG-3′; for mouse agouti-related protein (AGRP), sense 5′-CAG ACC GAG CAG AAG AAG-3′, antisense, 5′-GAC TCG TGC AGC CTT ACA-3′; for mouse ghrelin, sense, 5′-GAA AGG AAT CCA AGA AGC CA-3′, antisense, 5′-GCT TGA TGC CAA CAT CGA A-3′; for mouse agouti-related peptide (AGRP), sense, 5′-CAG ACC GAG CAG AAG AAG-3′, antisense, 5′-GAC TCG TGC AGC CTT ACA-3′; and for mouse β-actin, sense, 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′, antisense, 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′. The relative amount of mRNA was calculated with β-actin mRNA as the invariant control. The data are shown as the percentage of mean values of the control group which received saline.

Plasma active ghrelin assay. Plasma active ghrelin levels were measured by enzyme-linked immunosorbent assay (ELISA; Active Ghrelin ELISA kit, Mitsubishi Kagaku Iatron, Japan) according to the manufacturer's instructions. For the ELISA of active ghrelin, 1 N hydrochloric acid was added to the samples at a final concentration of 0.1 N immediately after plasma separation.

Statistical methods. Data are presented as mean values ± SEM. Statistical significance of differences between two groups was determined using Student's t test. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Hypothalamic 5-HT1B receptor and 5-HT2C receptor mRNA levels in fed and fasted mice

The hypothalamic 5-HT1B receptor mRNA levels and 5-HT2C receptor mRNA levels were significantly increased in 24-h fasted mice compared with fed mice (2-fold and 2-fold, respectively; Fig. 1A). Plasma active ghrelin levels were also significantly increased in 24-h fasted mice compared with fed mice (2.5-fold; Fig. 1B). These results indicate that increases in the expression of hypothalamic 5-HT1B receptor and 5-HT2C receptor genes are

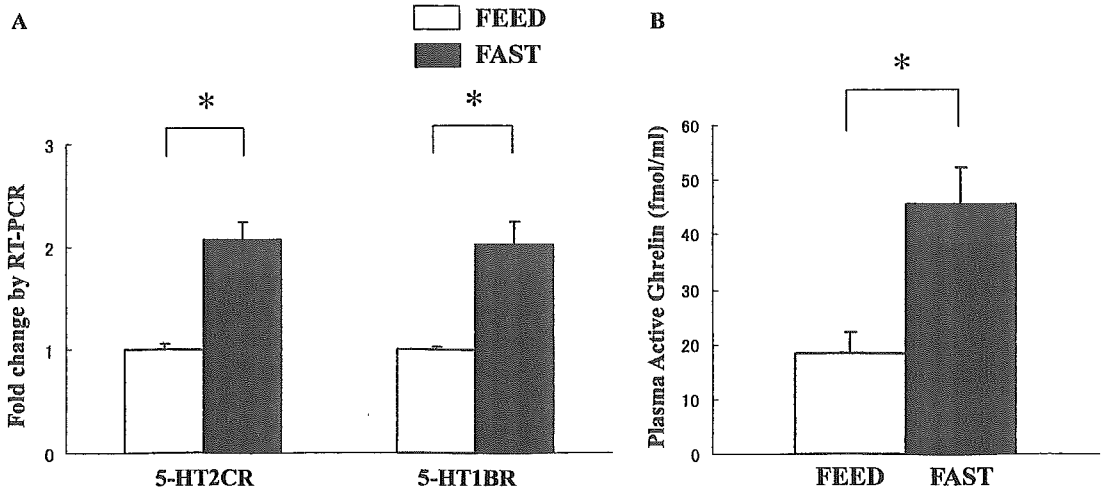

Fig. 1. Hypothalamic 5-HT2C receptor and 5-HT1B receptor mRNA levels (A) and plasma active ghrelin levels (B) in fed and 24-h fasted C57BL6J mice. Each column and bar represents the mean value ± SEM of six mice. *$P < 0.05$.

proportional to the increases in plasma active ghrelin levels after a 24-h fast.

Effects of mCPP or fenfluramine on plasma active ghrelin levels

To determine the effects of activation of brain 5-HT systems on plasma active ghrelin levels, we examined the effects of mCPP or fenfluramine on plasma levels of active ghrelin. Administration of either mCPP or fenfluramine significantly inhibited plasma active ghrelin levels after a 24-h fast (to 56% and 59% that of saline controls, respectively) (Fig. 2). These findings indicate that brain 5-HT systems via 5-HT2C receptors and/or 5-HT1B receptors play an inhibitory role in the regulation of plasma active ghrelin levels.

Effects of mCPP or fenfluramine on the expression of hypothalamic genes involved in the regulation of energy homeostasis

To determine the effects of mCPP or fenfluramine on the expression of the hypothalamic genes involved in the regulation of energy homeostasis, we examined the expression of hypothalamic POMC, CART, NPY, AGRP, and ghrelin genes that have an important role in the regulation of energy homeostasis [10,13,14]. mCPP slightly but significantly increased hypothalamic POMC and CART mRNA levels compared with saline controls (1.3- and 1.6-fold, respectively) while it had no significant effects on hypothalamic NPY, AGRP, and ghrelin mRNA levels (Fig. 3A). Fenfluramine significantly increased hypothalamic POMC and CART mRNA levels compared with saline controls (1.8- and 1.7-fold, respectively; Fig. 3B) while it had no significant effects on hypothalamic NPY, AGRP, and ghrelin mRNA levels (Fig. 3B). These results indicate that mCPP and fenfluramine increase the expression of hypothalamic POMC and CART genes without affecting the expression of hypothalamic NPY, AGRP, and ghrelin genes, and that

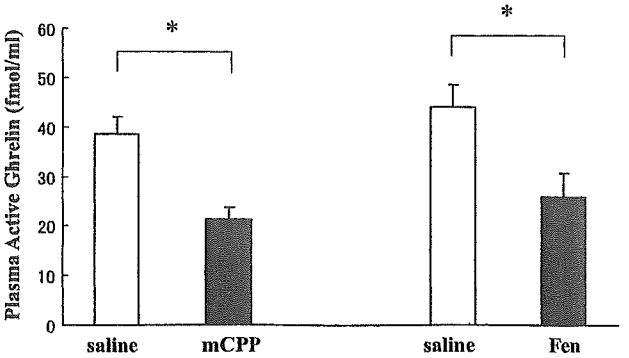

Fig. 2. Effects of mCPP (5 mg/kg) or fenfluramine (3 mg/kg) on plasma active ghrelin levels in C57BL6J mice. mCPP, fenfluramine, and saline were administered as described in Materials and methods. Each column and bar represents the mean value ± SEM of 8–12 mice. mCPP, *m*-chlorophenylpiperazine; Fen, fenfluramine. $^*P < 0.05$.

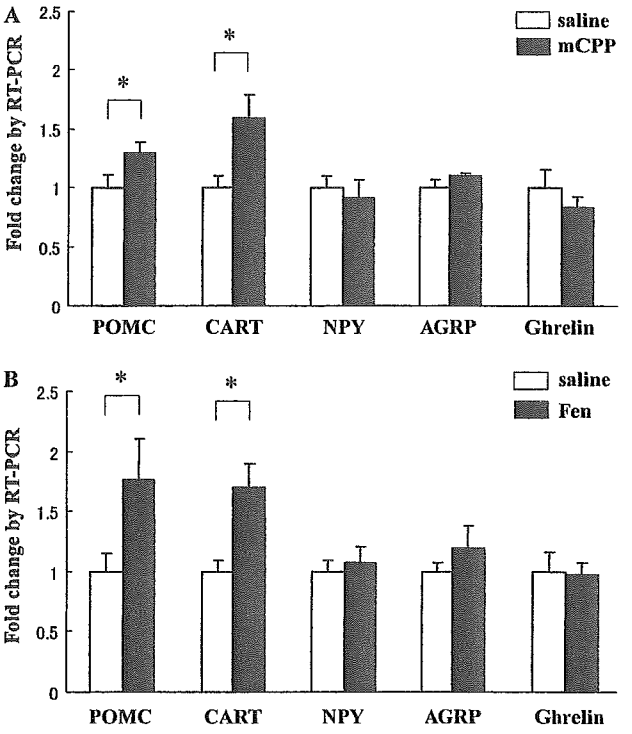

Fig. 3. Effects of mCPP (5 mg/kg) (A) or fenfluramine (3 mg/kg) (B) on hypothalamic POMC, CART, NPY, AGRP, and ghrelin mRNA levels in C57BL6J mice. mCPP, fenfluramine, and saline were administered as described in Materials and methods. Each column and bar represents the mean values ± SEM of five mice. POMC, pro-opiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; NPY, neuropeptide Y; AGRP, agouti-related protein; mCPP, *m*-chlorophenylpiperazine; Fen, fenfluramine. $^*P < 0.05$.

mCPP increases hypothalamic CART gene expression more than POMC gene expression.

The present results of our study demonstrate that 24-h fasting increases the expression of hypothalamic 5-HT1B receptor and 5-HT2C receptor genes associated with increases in plasma active ghrelin levels, and that mCPP and fenfluramine, which exert the anorexic effects via 5-HT2C receptors and/or 5-HT1B receptors, inhibit the increases in plasma active ghrelin levels induced by fasting. These results indicate that there is be a negative feedback system between brain 5-HT systems via 5-HT2C receptors and/or 5-HT1B receptors and plasma ghrelin levels in the regulation of energy homeostasis in mice.

Ghrelin is localized to the hypothalamic arcuate nucleus (ARC) and the stomach [10,15,16]. In the hypothalamus, ghrelin neurons contact the cell bodies and dendrites of NPY/AGRP and POMC neurons [10,16]. Hypothalamic NPY has been shown to mediate the hyperphagic action of ghrelin [17]. However, our present results demonstrate that either mCPP or fenfluramine inhibits plasma active ghrelin levels without altered the expression of hypothalamic NPY, AGRP, and ghrelin gene. Because 5-HT2C receptors are located in the central nervous system, but not the stomach [1,18], the inhibitory effects of mCPP and fenfluramine on plasma active ghrelin levels might be

mediated by the central 5-HT system. Direct effects of 5-HT on ghrelin secretion by the stomach, however, cannot be completely ruled out.

POMC neurons have been shown to express 5-HT2C receptors in the ARC [19]. POMC neurons are depolarized in response to either fenfluramine or mCPP in coronal slices of the hypothalamus of transgenic mice expressing green fluorescent protein under control of the POMC promoter [19]. The present results demonstrate that both fenfluramine and mCPP significantly increase the expression of hypothalamic POMC gene, although fenfluramine increases it more than mCPP. However, all of 5-HT2C receptors are not expressed on POMC neurons, although some of them are expressed on POMC neurons in the ARC [19]. Moreover, 5-HT2C receptors are expressed in a variety of hypothalamic nuclei other than the ARC that are involved in the regulation of feeding and energy homeostasis [18]. Accordingly, not all signals induced by the activation of 5-HT2C receptors are transferred to the melanocortin pathway.

The genes for CART, an anorexigenic peptide, are expressed in the hypothalamus, including the ARC, paraventricular nucleus, lateral hypothalamus, supraoptic nucleus, and dorsomedial nucleus of the hypothalamus [20–22]. The present results demonstrate that either mCPP or fenfluramine induces an increase in the expression of the hypothalamic CART gene, the downstream pathway of which is different from the melanocortin pathway [21–23]. Not only POMC but also CART neurons in the hypothalamus might therefore contribute to the inhibition of plasma active ghrelin levels induced by mCPP or fenfluramine.

In summary, these results suggest that there is a negative feedback system between brain 5-HT systems via 5-HT2C and/or 5-HT1B receptors and plasma active ghrelin levels, and that mCPP or fenfluramine increases the expression of hypothalamic POMC and CART genes without altering the expression of hypothalamic NPY, AGRP, and ghrelin genes in mice. This is the first report of interactions between brain 5-HT systems and plasma active ghrelin, and of the effects of activation of 5-HT systems on POMC, CART, NPY, AGRP, and ghrelin gene expression in the hypothalamus in vivo.

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Cell type-specific activation of metabolism reveals that β-cell secretion suppresses glucagon release from α-cells in rat pancreatic islets

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Divisions of [1]Molecular Metabolism and Diabetes and [2]Advanced Therapeutics for Metabolic Diseases, Tohoku University Graduate School of Medicine, Sendai, Miyagi; and [3]Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Mitaka, Tokyo, Japan

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Takahashi, Rui, Hisamitsu Ishihara, Akira Tamura, Suguru Yamaguchi, Takahiro Yamada, Daisuke Takei, Hideki Katagiri, Hitoshi Endou, and Yoshitomo Oka. Cell type-specific activation of metabolism reveals that β-cell secretion suppresses glucagon release from α-cells in rat pancreatic islets. *Am J Physiol Endocrinol Metab* 290: E308–E316, 2006. First published September 27, 2005; doi:10.1152/ajpendo.00131.2005.—Abnormal glucagon secretion is often associated with diabetes mellitus. However, the mechanisms by which nutrients modulate glucagon secretion remain poorly understood. Paracrine modulation by β- or δ-cells is among the postulated mechanisms. Herein we present further evidence of the paracrine mechanism. First, to activate cellular metabolism and thus hormone secretion in response to specific secretagogues, we engineered insulinoma INS-1E cells using an adenovirus-mediated expression system. Expression of the Na^+-dependent dicarboxylate transporter (NaDC)-1 resulted in 2.5- to 4.6-fold ($P < 0.01$) increases in insulin secretion in response to various tricarboxylic acid cycle intermediates. Similarly, expression of glycerol kinase (GlyK) increased insulin secretion 3.8- or 4.2-fold ($P < 0.01$) in response to glycerol or dihydroxyacetone, respectively. This cell engineering method was then modified, using the Cre-*loxP* switching system, to activate β-cells and non-β-cells separately in rat islets. NaDC-1 expression only in non-β-cells, among which α-cells are predominant, caused an increase (by 1.8-fold, $P < 0.05$) in glucagon secretion in response to malate or succinate. However, the increase in glucagon release was prevented when NaDC-1 was expressed in whole islets, i.e., both β-cells and non-β-cells. Similarly, an increase in glucagon release with glycerol was observed when GlyK was expressed only in non-β-cells but not when it was expressed in whole islets. Furthermore, dicarboxylates suppressed basal glucagon secretion by 30% ($P < 0.05$) when NaDC-1 was expressed only in β-cells. These data demonstrate that glucagon secretion from rat α-cells depends on β-cell activation and provide insights into the coordinated mechanisms underlying hormone secretion from pancreatic islets.

pancreatic islet; paracrine regulation; glucagon secretion; cell activation

PANCREATIC ISLETS OF LANGERHANS play a central role in glucose homeostasis. In diabetic patients, not only insulin but also glucagon secretion is impaired. Basal levels of serum glucagon are elevated, and a rise in blood glucose fails to inhibit, and can paradoxically even stimulate, glucagon release in subjects with diabetes (14). In addition, the glucagon secretory response is impaired when circulating glucose drops (11), which may result in life-threatening hypoglycemia in patients treated with insulin. This unresponsiveness to hypoglycemia makes precise glycemic management difficult (8), although strict control is known to be essential for preventing diabetic complications (38). Therefore, it is important to understand the mechanisms whereby glucagon secretion is regulated by nutrients.

Three types of regulatory mechanisms have been proposed by which nutrients, such as glucose, suppress glucagon secretion. The first is a direct action of glucose on α-cells (16, 23). Glucose metabolism in α-cells is considered to generate signals that inhibit glucagon secretion, whereas glucose metabolism increases insulin secretion in β-cells. Therefore, intracellular signaling arising from glucose metabolism might differ between the two cell types, although α-cells also express molecules essential for stimulus-secretion coupling in β-cells, including ATP-sensitive K^+ (K_{ATP}) channels (3, 5). The second mechanism involves modulation by neighboring endocrine cells, such as β- (2, 10, 13, 17, 33, 39) and δ-cells (7, 34). Several molecules, including insulin (2, 13, 33), Zn^{2+} (10, 17), γ-aminobutyric acid (GABA; see Ref. 39), and somatostatin (7, 34), have been postulated to be mediators of these inhibitory effects. Autonomic regulation is the third mechanism (6, 37) and might be clinically important for responses to hypoglycemia, although in humans the glucagon response to hypoglycemia from a transplanted (denerved) pancreas is intact, arguing against this possibility (9).

Studies of stimulus-secretion coupling in β-cells, the predominant cell type of islets, have made great progress in recent decades (20). In contrast, α-cell research has been hampered because of difficulties in getting sufficient numbers of this cell type. Nonetheless, an earlier study has found important characteristics of α-cells (32), and several recent studies have discovered interesting features of this cell type. Characterization of electrical activity and calcium dynamics revealed a unique ion channel composition in α-cells (12). In addition, pyruvate induces glucagon secretion from α-cells (17) but does not stimulate insulin secretion from β-cells. This is probably because α-cells have a transporting system for pyruvate but β-cells do not. This observation suggests that metabolized nutrients can induce exocytosis in α-cells as is the case in β-cells. However, when the pancreas is perfused or islets are stimulated with metabolized nutrients such as glucose, insulin secretion is stimulated, whereas glucagon secretion is suppressed.

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In this study, to gain insight into the regulatory mechanism governing glucagon secretion in islets, we have established a method to activate cellular metabolism in β-cells and non-β-cells separately. For this purpose, we have expressed Na^+-dependent dicarboxylate transporter (NaDC)-1 or glycerol kinase (GlyK) in β-cells and/or non-β-cells. Using this method, we showed rat α-cells to secret glucagon when metabolically activated in the absence of β-cell activation. In addition, basal glucagon secretion was shown for the first time to be suppressed by β-cell activation. These data contribute to our understanding of the regulation of islet hormone secretion, providing insights that are anticipated to be of value in managing hypoglycemia and hyperglycemia in subjects with diabetes.

MATERIALS AND METHODS

Generation of recombinant adenoviruses bearing rat GlyK cDNA (AdRIPHAGlyK and AdCAGlxHAGlyKlx) and NaDC-1 cDNA (AdRIPNaDC and AdCAGlxNaDClx). Rat GlyK cDNA (31) was amplified using rat liver total RNA. An entire coding region was sequenced and subcloned downstream of the hemagglutinin (HA)-epitope sequence. Rat NaDC-1 cDNA was as described previously (36). A *Sph*I-*Spe*I fragment of HA-tagged GlyK (HAGlyK) cDNA and a *Sal*I-*Sma*I fragment of NaDC-1 cDNA were ligated between the 410-bp fragment of the rat insulin 1 promoter and the rabbit β-globin poly(A) signal region. The resulting expression units were used for generation of AdRIPHAGlyK and AdRIPNaDC by the methods described previously (27). Rat GlyK and NaDC-1 cDNA were also subcloned between two *lox*P sequences and ligated under the CAG (a transcriptional unit composed of the cytomegalovirus enhancer, the actin promoter, and the globin intron) promoter unit (28). Recombinant viruses harboring these expression units were then generated (AdCAGlxHAGlyKlx and AdCAGlxNaDClx). AdCAGlacZ (27) expressing β-galactosidase was used as a control adenovirus. AdRIPNCre was renamed from AdInsPNCre generated as described previously (17). Adenovirus titers were measured by the method described previously (27).

Isolation of rat islets and infection with recombinant adenoviruses. Rat islets were prepared by retrograde collagenase infusion through the common bile duct and hand picked under the microscope. Isolated islets were infected with the recombinant adenoviruses at 1.2×10^6 plaque-forming units (PFU)/islet in 1.0 ml medium for 60 min. In the case of combined infection of AdCAGlxNaDClx plus AdRIPNCre or AdCAGlxHAGlyKlx plus AdRIPNCre, the amount of AdRIPNCre was four times greater than the others, with a total amount of 1.2×10^6 PFU/islet.

Immunoblot analysis. INS-1E cells (25) were infected with either AdRIPHAGlyK or AdRIPNaDC at multiplicity of infection (MOI) of 100, cultured for 2 days, and directly dissolved in the SDS sample buffer. Proteins were subjected to SDS-PAGE and were transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-rat NaDC-1 antibody raised against the carboxy-terminal peptide (1:500; see Ref. 36) or with anti-HA tag antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and then incubated for 1 h with anti-rabbit IgG (1:1,000) conjugated with horseradish peroxidase, respectively. Detection was accomplished with chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

Immunocytochemical analyses. INS-1E cells infected with either AdRIPHAGlyK or AdRIPNaDC at an MOI of 100 were incubated with anti-rat NaDC-1 antibody (1:500) or with anti-HA tag antibody (1:200) for 1 h at room temperature and then incubated for 1 h with FITC-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA). Islets infected with AdRIPHAGlyK, AdCAGlxHAGlyKlx alone, or AdCAGlxHAGlyKlx plus AdRIPNCre were dispersed on coverslips. Cells were then fixed with 4% paraformaldehyde and incubated with anti-HA tag antibody (1:200) followed by incubation with FITC-conjugated anti-rabbit IgG. Insulin and glucagon were also stained using mouse monoclonal antibodies against these hormones (1:1,000; Sigma-Aldrich, Tokyo, Japan) and Texas red-conjugated anti-mouse IgG (1:500; Jackson ImmunoResearch).

Hormone secretion. INS-1E cells (0.2×10^6 cells/well of 24-well plates) or islets (10 islets/tube) infected with recombinant adenoviruses were incubated over a period of 60 min in 1 ml of Krebs-Ringer-bicarbonate-HEPES buffer [140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES (pH 7.4), and 0.1% BSA] containing 2.5 mM glucose plus indicated stimulators. Insulin and glucagon were detected by RIA kits (Linco, St. Louis, MO).

Statistical analyses. Data are presented as means ± SE. Differences between groups were assessed by Student's *t-test* for unpaired data.

RESULTS

Expression of NaDC-1 resulted in cell activation in response to dicarboxylates. We first sought to establish a means of activating metabolism in specific cell types of pancreatic islets to study *1*) the roles of α-cell nutrient metabolism in glucagon secretion and *2*) whether activation of neighboring β-cells in response to nutrient metabolism modulates α-cell secretion. It was previously shown that β-cells expressing monocarboxylate transporter (MCT-1) metabolize pyruvate and secrete insulin in response to the monocarboxylate (18). Similarly, insulin secretion is reportedly stimulated in β-cells expressing GlyK in response to glycerol (1, 29). These data suggested that cells normally unresponsive to some nutrients can be activated by expressing protein(s) needed for their metabolism. We tested whether tricarboxylic acid (TCA) cycle intermediates alter insulin and glucagon secretion in isolated rat islets and found α-ketoglutarate, succinate, fumarate, and malate to have no effects on hormone secretion in wild-type islets (Fig. 1). A membrane-permeable analog of succinate, methylsuccinate, is known to stimulate insulin secretion (24), suggesting that inability of TCA cycle intermediates to activate β-cells is attributable to low or no expression of membrane transporters for these compounds. Therefore, to activate cells, a recombinant adenovirus harboring cDNA encoding rat NaDC-1 under the rat insulin promoter (AdRIPNaDC) was constructed, with the aim of activating the cells with TCA cycle intermediates.

We first employed rat insulinoma INS-1E cells to study whether the recombinant adenovirus induces functional expression of NaDC-1, leading to increased cellular metabolism and thereby promoting insulin secretion. Western blotting using an antibody against NaDC-1 showed strong expression of NaDC-1, with the expected protein size, in insulinoma INS-1E cells infected with AdRIPNaDC (Fig. 2*A*). Immunocytochemical analysis revealed strong staining at the cell surface, although weak staining was observed inside the cell, suggesting improper targeting of some expressed membrane proteins because of forced expression (Fig. 2*B*). As shown in Fig. 2*C*, adenovirus-mediated expression of NaDC-1 made INS-1E cells responsive to various TCA cycle intermediates. Citrate, one of the tricarboxylates, existing partly in a divalent form at pH 7.4 (36), could be transported into INS-1E cells expressing NaDC-1, and thereby induced insulin secretion. A nonmetabolizable dicarboxylate, glutarate, failed to induce insulin secretion from INS-1E cells expressing NaDC-1, indi-

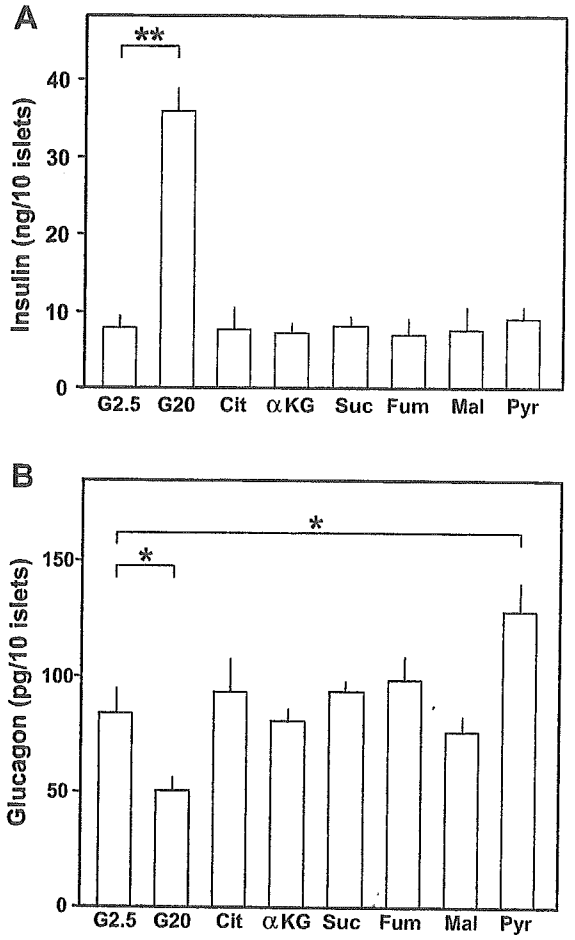

Fig. 1. Tricarboxylic acid (TCA) cycle intermediates had no effects on either insulin or glucagon secretion. Isolated islets were cultured overnight and challenged with glucose (20 mM), pyruvate (10 mM), and various TCA cycle intermediates (10 mM). Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured. G2.5, 2.5 mM glucose; G20, 20 mM glucose; Cit, citrate; αKG, α-ketoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; Pyr, pyruvate. Data are means ± SE; $n = 3\sim7$. $^*P < 0.05$ and $^{**}P < 0.01$.

cating insulin secretion evoked by dicarboxylates to be the result of activation of metabolism. In addition, insulin secretion evoked by malate was abolished by 2 mM NaN_3, a metabolic inhibitor (data not shown), further supporting this notion.

Expression of GlyK resulted in cell activation in response to glycerol. We also expressed rat GlyK to activate the cellular glycolytic pathway. Glycerol and dihydroxyacetone can enter the glycolytic pathway after GlyK-mediated conversion to glycerol 3-phosphate and dihydroxyacetone phosphate, respectively. Adenovirus-mediated introduction of HAGlyK cDNA resulted in expression of this protein in the INS-1E cell cytosol (Fig. 2, *D* and *E*). Expressed HAGlyK was functional, since INS-1E cells expressing HAGlyK secreted insulin in response to glycerol or dihydroxyacetone (Fig. 2*F*), as was reported in INS-1E cells expressing *Escherichia coli* GlyK (1, 29).

Taken together, these data indicate NaDC-1 and GlyK expressions to be effective in activating cellular metabolism in response to certain nutrients.

Cell type-specific expressions of genes in isolated islets. To study the stimulus-secretion coupling in α-cells and possible cross-talk with other pancreatic endocrine cells, we next sought to express the genes of interest in α- and β-cells separately. As was reported previously (17), the rat insulin 1 promoter has high transcription activity and specificity for β-cell-restricted expression of foreign genes. Therefore, β-cell-specific expression of NaDC-1 or HAGlyK was achieved using recombinant adenovirus vectors with the rat insulin 1 promoter (Fig. 3*A*). When islets were infected with AdRIPHAGlyK, >60% of insulin-positive cells were stained with HA (Fig. 3*D*), but none of the glucagon-positive cells expressed HAGlyK (Fig. 3*E*).

In contrast to the insulin 1 promoter for β-cells, the glucagon promoter (1.6 kbp) did not have high transcriptional activities specific for α-cells when placed in the adenoviral genome (data not shown). To increase expression in α-cells, a dual-adenovirus approach was previously developed (17); one adenovirus produces Cre recombinase under the glucagon promoter, and the other virus expresses the desired genes under the potent CAG promoter unit (28) once the intervening sequence is excised by Cre recombinase (17). Although the strategy increased the expression levels of reporter genes, such as luciferase, in α-cells, it did not significantly increase the activities of cellular enzymes or transporters, such as glucokinase and MCT-1 (data not shown). We then raised the recombinant virus titer. However, a high titer of virus with the glucagon promoter also induced expression in cells other than α-cells (data not shown). We therefore did not employ the glucagon promoter in this study but rather devised a method employing the insulin 1 promoter and the Cre-*loxP* system.

As shown in Fig. 3*C*, a cDNA floxed with *loxP* sequences was placed downstream from the CAG promoter unit (28) that enables transcription in any cell type. This expression unit was then introduced into islet cells, together with the insulin promoter-Cre adenovirus (AdRIPNCre; see Ref. 17). The cDNA was expected to be removed from the unit by the Cre recombinase in the β-cell, allowing expression of the genes of interest in non-β-cells, a cell population where α-cells are predominant. Indeed, when rat islets were infected with AdCAGlxHAGlyKlx and AdRIPNCre, ~70% of glucagon-positive cells was stained with HA (Fig. 3, *H* and *I*). More than 80% of HA-positive cells were observed to be stained with glucagon, and <10% were insulin positive, although HA staining was occasionally observed in somatostatin-positive cells and fibroblast-like cells (data not shown). When islets were infected with AdCAGlxHAGlyKlx alone (Fig. 3*B*), ~60% of β-cells (Fig. 3*F*) and 65% of α-cells (Fig. 3*G*) expressed HAGlyK.

α-Cell activation triggered glucagon secretion when β-cells remained nonactivated. To study the role of nutrient metabolism in glucagon secretion from α-cells, isolated rat islets were infected with AdCAGlxNaDClx plus AdRIPNCre and challenged with succinate or malate. As shown in Fig. 4, *A* and *B*, glucagon secretion was increased by 80%, without changes in insulin secretion. These effects were abolished by 2 mM NaN_3, indicating the observed glucagon secretion to be due to activation of cellular metabolism of the dicarboxylates. When islets were infected with AdCAGlxHAGlyKlx plus AdRIPNCre and then challenged with 10 mM glycerol, insulin secretion did not change (Fig. 4*C*) and glucagon secretion tended to increase, but the differences did not reach statistical significance (Fig. 4*D*).

In contrast, when α-cells were activated together with β-cells by infecting islets with AdCAGlxNaDClx alone, i.e., without AdRIPNCre (Fig. 5, *A* and *B*), insulin secretion was increased by more than threefold in response to 10 mM malate

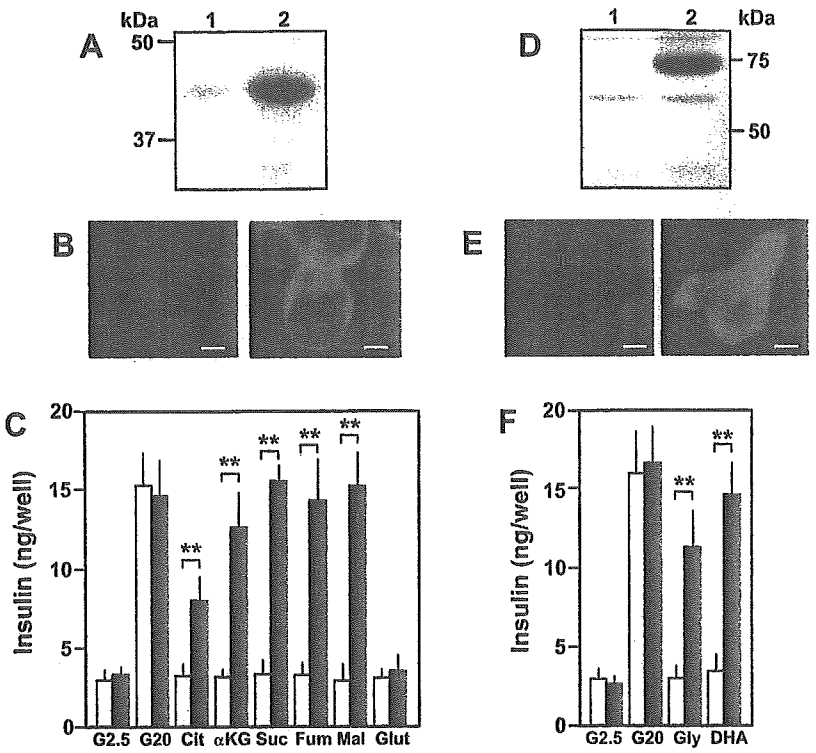

Fig. 2. Adenovirus-mediated Na^+-dependent dicarboxylate transporter (NaDC)-1 or glycerol kinase (GlyK) expression in INS-1E cells. *A*: INS-1E cells infected with either AdCAGlacZ (*lane 1*) or AdRIPNaDC (*lane 2*) were subjected to SDS-PAGE and probed with an anti-NaDC-1 antibody. *B*: INS-1E cells infected with either AdCAGlacZ (*left*) or AdRIPNaDC (*right*) were stained with anti-NaDC-1 antibody. Bars, 4 μm. *C*: INS-1E cells infected with either AdCAGlacZ (open bars) or AdRIPNaDC (filled bars) were challenged with 20 mM glucose or various TCA cycle intermediates (10 mM). Insulin secreted during a 60-min incubation was measured. Data are means ± SE; $n = 5$. $^{**}P < 0.01$. Glut, glutarate. *D*: INS-1E cells infected with either AdCAGlacZ (*lane 1*) or AdRIPHAGlyK (*lane 2*) were subjected to SDS-PAGE and probed with an anti-hemagglutinin (HA) antibody. *E*: INS-1E cells infected with either AdCAGlacZ (*left*) or AdRIPHAGlyK (*right*) were stained with an anti-HA antibody. Bars, 4 μm. *F*: INS-1E cells infected with either AdCAGlacZ (open bars) or AdRIPHAGlyK (filled bars) were challenged with 20 mM glucose, 10 mM glycerol (Gly), or 10 mM dihydroxyacetone (DHA). Insulin secreted during a 60-min incubation was measured. Data are means ± SE; $n = 4$. $^{**}P < 0.01$.

or succinate, whereas glucagon release was unchanged. Similarly, AdCAGlxHAGlyKlx infection increased insulin (by 2.5-fold) but not glucagon secretion with a 10 mM glycerol challenge (Fig. 5, *C* and *D*). These data indicate that α-cell activation dose not lead to glucagon secretion when β-cells are activated simultaneously.

Recent studies have postulated several molecules, including insulin (2, 13, 33), Zn^{2+} (10, 17), and GABA (39), as mediators of β-cell inhibitory effects on glucagon secretion. During succinate stimulation, insulin secreted from β-cells expressing NaDC-1 amounted to ~25 ng/ml (Fig. 5*A*). We therefore examined whether this amount of insulin inhibits glucagon secretion from islets expressing NaDC-1 in α- but not β-cells. As shown in Fig. 6, succinate-stimulated glucagon secretion from islets infected with AdCAGlxNaDClx plus AdRIPNCre was significantly suppressed by 25 ng/ml insulin, indicating that insulin mediates inhibitory effects of β-cells on glucagon secretion. However, the suppression seemed incomplete, sug-

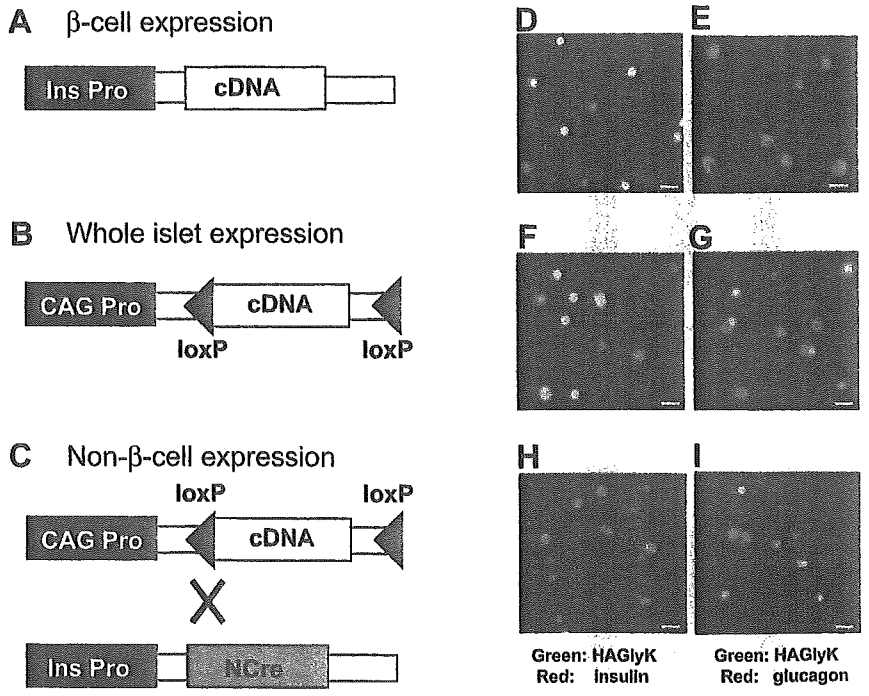

Fig. 3. Selective gene expression method for β- and non-β-cells in islets. *A–C*: schematic representation of adenoviruses for expression in β-cells (*A*), whole islet cells (*B*), and non-β-cells (*C*). NCre, nuclear targeted Cre recombinase; CAG, a transcriptional unit composed of the cytomegalovirus enhancer, the actin promoter, and the globin intron (28). *D* and *E*: islets infected with AdRIPHAGlyK were dispersed and stained with an anti-HA (green) antibody (*D* and *E*) together with anti-insulin (red; *D*) or anti-glucagon (red; *E*) antibody. *F* and *G*: islets infected with AdCAGlxHAGlyKlx alone were dispersed and stained with an anti-HA (green) antibody (*F* and *G*) together with anti-insulin (red; *F*) or anti-glucagon (red; *G*) antibody. *H* and *I*: islets infected with AdCAGlx-HAGlyKlx plus AdRIPNCre were dispersed and stained with an anti-HA (green) antibody (*H* and *I*) together with anti-insulin (red; *H*) or anti-glucagon (red; *I*) antibody. Bars, 10 μm. Colocalization resulted in yellow.

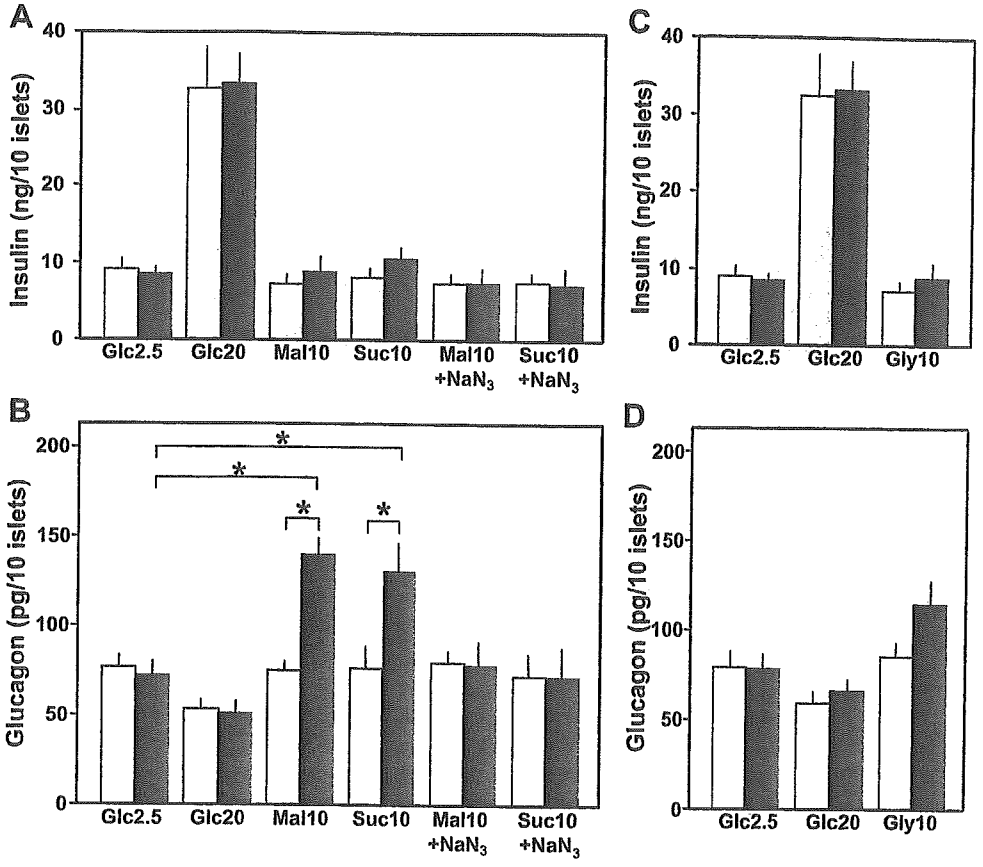

Fig. 4. Selective α-cell activation induced glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlx-NaDClx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, 10 mM malate (Mal10), or 10 mM succinate (Suc10) with or without 2 mM NaN_3. Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured; $n = 3\sim5$. $^*P < 0.05$. *C* and *D*: isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlxHAGlyKlx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, or 10 mM glycerol (Gly10). Insulin (*C*) and glucagon (*D*) secreted during a 60-min incubation were measured; $n = 4$.

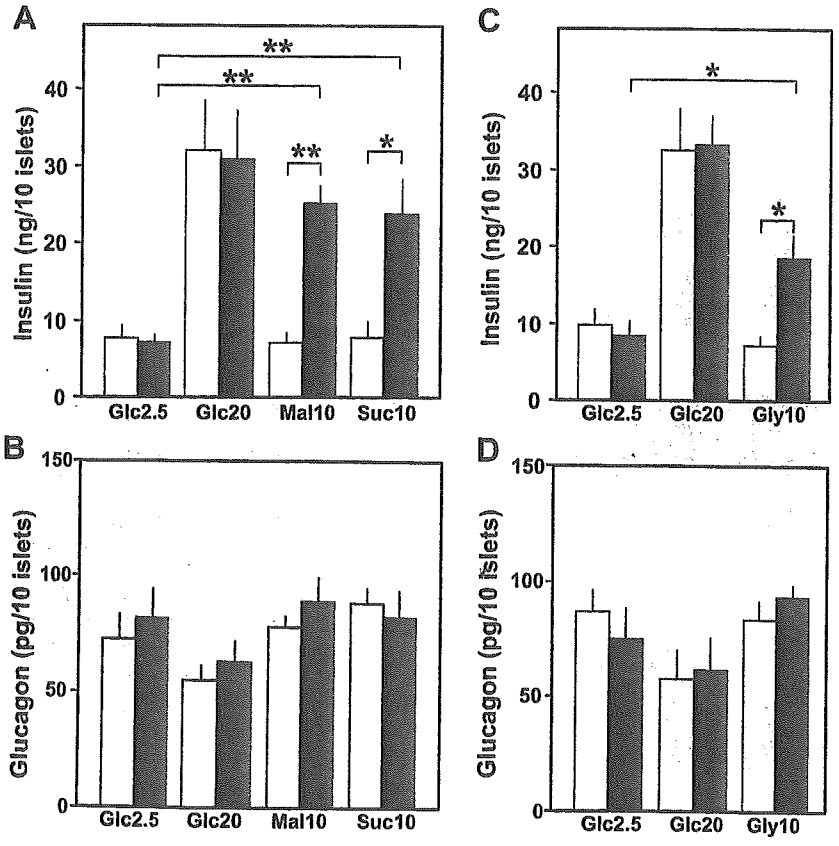

Fig. 5. Simultaneous α- and β-cell activation induced insulin but not glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdCA-GlxNaDClx (filled bars) were challenged with 20 mM glucose, 10 mM malate, or 10 mM succinate. Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured; $n = 4\sim6$. $^*P < 0.05$ and $^{**}P < 0.01$. *C* and *D*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdCAGlxHAGlyKlx (filled bars) were challenged with 20 mM glucose, or 10 mM glycerol. Insulin (*C*) and glucagon (*D*) secreted during a 60-min incubation were measured; $n = 4$. $^*P < 0.05$.

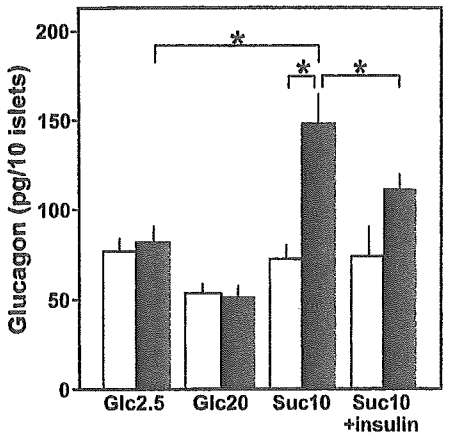

Fig. 6. Insulin suppressed succinate-stimulated glucagon secretion. Isolated islets (10 islets/tube) infected with AdCAGlacZ plus AdRIPNCre (open bars) or AdCAGlxNaDClx plus AdRIPNCre (filled bars) were challenged with 20 mM glucose, 10 mM succinate alone, or 10 mM succinate with 25 ng/ml insulin. Glucagon secreted during a 60-min incubation was measured; $n = 4$. $^*P < 0.05$.

gesting another molecule(s) is also important in suppression of glucagon secretion by β-cell activation.

β-Cell activation increased insulin secretion and decreased basal glucagon secretion. We next studied whether basal glucagon secretion was altered by β-cell activation. For this purpose, NaDC-1 was expressed only in β-cells by infecting islets with AdRIPNaDC. When these islets were challenged with 10 mM malate or succinate, insulin secretion more than doubled (Fig. 7*A*). Interestingly, glucagon secretion from the same islets was reduced significantly, by 30% (Fig. 7*B*). Similar modulation of hormone secretion was observed when islets were infected with AdRIPGlyK; insulin secretion was increased significantly, by 1.7-fold, in response to 10 mM glycerol (Fig. 7*C*), whereas glucagon secretion tended to decrease, although not to a statistically significant degree (Fig. 7*D*).

DISCUSSION

Abnormal nutrient-mediated modulation of glucagon secretion is often associated with diabetes mellitus. However, the mechanisms whereby nutrients modulate glucagon secretion remain poorly understood. Paracrine modulation by β- or δ-cells is among the postulated mechanisms. Herein we provide further evidence that glucagon secretion from α-cells is stimulated by nutrient metabolism in the absence of β-cell activation but it is suppressed when β-cells are activated.

We first demonstrated, employing NaDC-1 expression, that TCA cycle intermediates induce insulin secretion from β-cells engineered to transport these substrates. It was previously reported that pyruvate and lactate stimulate insulin secretion from β-cells expressing MCT-1 and lactate dehydrogenase (LDH) but not from normal β-cells (18). This was interpreted as low levels of MCT-1 and LDH expression protecting β-cells

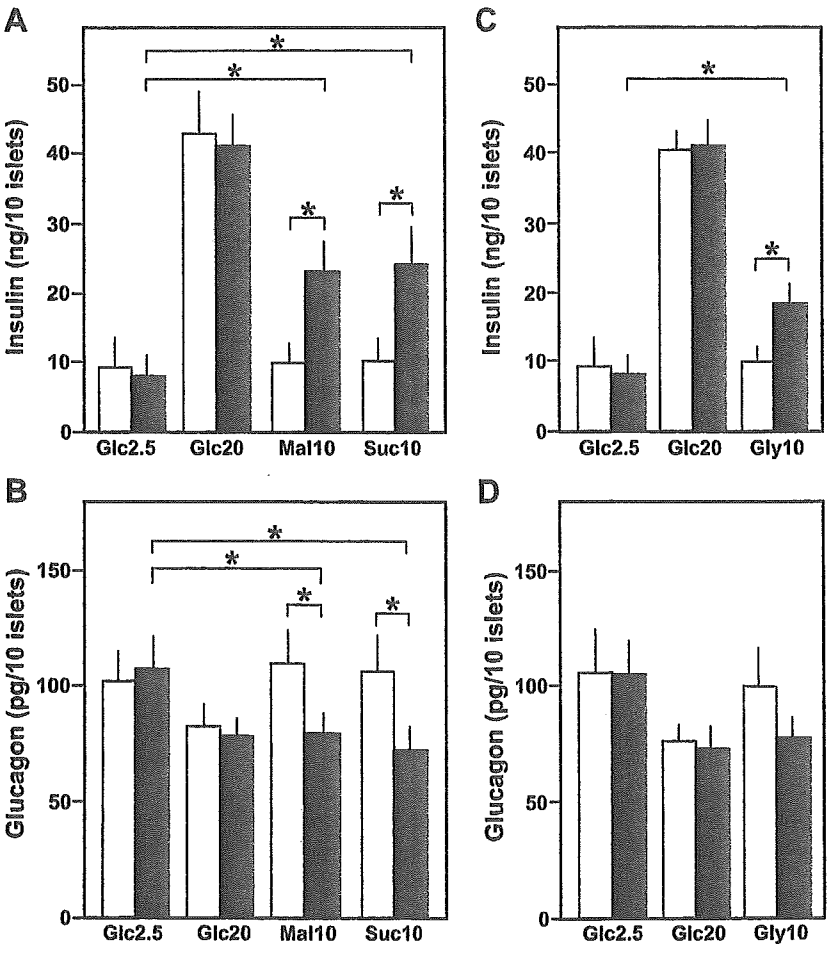

Fig. 7. Selective β-cell activation inhibited glucagon secretion. *A* and *B*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdRIPNaDC (filled bars) were challenged with 20 mM glucose, 10 mM malate, or 10 mM succinate. Insulin (*A*) and glucagon (*B*) secreted during a 60-min incubation were measured. $^*P < 0.05$; $n = 5$. *C* and *D*: isolated islets (10 islets/tube) infected with AdCAGlacZ (open bars) or AdRIPHAGlyK (filled bars) were challenged with 20 mM glucose or 10 mM glycerol. Insulin (*C*) and glucagon (*D*) secreted during a 60-min incubation were measured. $^*P < 0.05$; $n = 4$.

from the stimulatory effects of pyruvate and lactate, which could otherwise cause undesired insulin secretion in catabolic states, such as during exercise. Similarly, the present data suggest that low levels of NaDC-1 expression protect β-cells from the stimulatory effects of dicarboxylates and confer glucose selectivity on insulin secretion.

A major part of the present study was based on the method we devised to activate cellular metabolism in β-cells and non-β-cells, separately, in primary rat islets by specifically expressing NaDC-1 or GlyK in β-cells and non-β-cells. Employing this innovative approach, we showed TCA cycle intermediates, succinate and malate, to induce glucagon secretion when NaDC-1 is expressed in α-cells. Stimulation of glucagon secretion was previously demonstrated in intact islets challenged with another mitochondrial substrate, pyruvate, which exerts essentially no stimulatory effects on β-cells (17). In subjects with type 1 diabetes, glucose reportedly failed to suppress, or even slightly stimulated, glucagon secretion (14). Abnormal glucagon secretion in response to glucose was also reported in islets from insulin-deficient Chinese hamsters (19). The present data, obtained employing NaDC-1 expression, thus support the concept of α-cells having an inherent capacity to increase glucagon secretion in response to nutrients under certain circumstances, i.e., in the absence of β-cell effects. GlyK expression in α-cells tended to increase glucagon secretion in response to glycerol, but not to a statistically significant degree (Fig. 3*D*). This might be because the coupling of glycolysis and mitochondrial metabolism is less efficient in α-cells than in β-cells, as previously suggested (35). In contrast, dicarboxylates directly stimulate mitochondrial metabolism in cells expressing NaDC-1, thereby possibly producing a significant increase in glucagon secretion.

The concept of α-cells having an inherent capacity to increase glucagon secretion in response to nutrients has been reinforced recently by the demonstration of glucagon secretion stimulated by glucose from purified rat α-cells (10). An earlier study (32), however, reported that glucose inhibited glucagon secretion induced by an amino acid mixture in purified α-cells. Thus direct action on α-cells could be multiple, both inhibitory and stimulatory in nature. Glucose reportedly promotes the filling of the endoplasmic reticulum Ca^{2+} stores in α-cells (23) as in β-cells (20). In the presence of an amino acid mixture, glucose inhibitory effects could attenuate the rise in cytosolic Ca^{2+} induced by amino acids, whereas glucose stimulatory effects could be masked by amino acid-stimulated Ca^{2+} elevation.

Glucagon secretion stimulated by pyruvate was previously shown to be suppressed by activation of β-cells expressing MCT-1 (17). Similar inhibition of activated glucagon secretion by β-cell secretory activities was recently reported in β-cell-specific Foxa2 knockout mice (22). Islets from these mice secreted insulin in response to an amino acid mixture, and, interestingly, the glucagon secretion that is normally seen in the wild-type islets in response to amino acids was abolished in the mutant islets. This result is consistent with the notion that suppression of activated glucagon secretion is attributable to β-cell secretory activities. In the present study, for the first time, we have shown basal glucagon secretion to also be suppressed by β-cell activation. In addition, in NaDC-1-expressing cells, glucose stimulated insulin secretion more potently (an ~4.5-fold increase) than dicarboxylates (an ~2-fold increase; Fig. 7*A*), whereas glucose and dicarboxylates suppressed glucagon secretion to a similar extent (~30%). We speculated that this is because, when islets were challenged with glucose, α-cells were also activated for glucagon secretion, which counteracted the suppressing effect exerted by β-cell secretory activities. Recent studies demonstrated that insulin (2, 13, 33), Zn^{2+} (10, 17), and GABA (39) are candidates for β-cell-derived inhibitory substances of glucagon secretion in rat islets. Our observation of inhibitory effects of insulin on succinate-stimulated glucagon secretion from islets expressing NaDC-1 in α- but not β-cells supports this notion about the role of insulin. To study roles of Zn^{2+} and GABA, it is crucial to determine amounts of these molecules secreted from β-cells during nutrient stimulation. Further studies are needed to elucidate the molecular basis of β-cell inhibitory effects.

Glucagon secretion was reported to depend differentially on Ca^{2+} influx through N- and L-type Ca^{2+} channels (12, 16). N-type Ca^{2+} channels operate predominantly under basal conditions and L-type Ca^{2+} channels in the stimulated state. β-Cell activation suppressed glucagon secretion regardless of whether α-cells were in the basal (Fig. 7*B*) or the stimulated state (Fig. 4*B*; see Refs. 17 and 22), suggesting the suppressed glucagon secretion to possibly be due to direct inhibition of two Ca^{2+} channels or to indirect inhibition of Ca^{2+} channels resulting from prevention of membrane depolarization. The latter could be achieved by opening of $GABA_A$ receptor Cl^- channels in the α-cell (39). In addition, prevention of membrane depolarization is also brought about by activation of K_{ATP} channels, which is reportedly induced by the β-cell secretory products, Zn^{2+} (4, 10) and insulin (10, 21). However, involvement of K_{ATP} channels in regulating glucagon secretion is controversial, since different glucagon responses were demonstrated in the following two mutant islets lacking functional K_{ATP} channels: preserved glucagon responses from islets deficient in one of the K_{ATP} channel subunits, Kir6.2 (26), and no response from islets deficient in another subunit, sulfonylurea receptor 1 (16).

Although inhibition of glucagon secretion by activation of β-cells expressing NaDC-1 supports the paracrine mechanism, it does not exclude a direct inhibitory effect of glucose metabolism on glucagon secretion, especially at relatively low glucose concentrations and in the presence of stimulators of glucagon secretion, such as an amino acid mixture (see above). Two different mechanisms by which glucose directly suppresses glucagon secretion have been proposed. One involves a store-operated current, which controls a depolarizing cascade leading to opening of L-type Ca^{2+} channels in α-cells (23). Thus glucose-induced ATP generation stimulates Ca^{2+} sequestration in endoplasmic reticulum and modulates a store-operated current. Another is based on low K_{ATP} channel activity and the special ion channel composition of the α-cell (5, 15); K_{ATP} channel closure by ATP produced during glucose metabolism causes modest depolarization, which inactivates, instead of activating, voltage-gated Na^+, T- and N-type Ca^{2+}, and A-type K^+ channels participating in action potential generation. Both models are based on data obtained in mouse α-cells, in which the K_{ATP} channel density is much less than

that in rat α-cells. Rat α-cells were calculated to have nearly 100-fold more K_{ATP} channels than mouse α-cells and double the number in rat β-cells (3, 5). K_{ATP} channels couple nutrient metabolism to membrane depolarization. Therefore, in rat α-cells with a greater number of K_{ATP} channels, nutrient metabolism could induce greater changes in membrane potential compared with those in mouse α-cells, thereby allowing glucagon secretion. Thus the importance of paracrine inhibition might be species dependent. It is essential to establish the level of K_{ATP} channel expression in human α-cells and whether this channel contributes to the regulation of glucagon secretion in humans. In this context, it is noteworthy that K_{ATP} channel-blocking agents stimulated glucagon secretion in subjects with insulin-deficient type 1 diabetes (30).

In summary, our findings provide further evidence supporting the concept that α-cell exocytosis can be modulated by β-cells via a paracrine mechanism. Future studies should focus on detailed molecular analyses of stimulus-secretion coupling in α-cells under paracrine regulation. This is a promising approach to identifying new drug targets for treating α-cell abnormalities in diabetic patients.

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