Original Paper

AMP010014A09 in *Sus Scrofa* Encodes an Analog of G Protein-Coupled Receptor 109A, Which Mediates the Anti-Inflammatory Effects of Beta-Hydroxybutyric Acid

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Key Words

AMP+ • GPCRs • IPEC-J2 • Monocytes • ST • Swine

Abstract

Background: Hydroxy-carboxylic acid receptor 2 (HCA₂, also called GPR109A) belongs to the G protein-coupled receptor (GPCR) family and is found in humans, rats, mice, hamsters and guinea pigs, but there are almost no reports of this protein in other species. In this investigation, we speculated that AMP010014A09 (AMP+) is a homologue of GPR109A in swine. **Methods:** To test this hypothesis, the following experiments were designed: monocytes isolated from the peripheral blood of swine were treated with LPS after pretreating with or without β -hydroxybutyric acid (BHBA), and the levels of pro-inflammatory cytokines and inflammatory proteins were assessed. cAMP levels induced by Forskolin in swine testicular (ST) and IPEC-J2 cells were detected with or without BHBA treatment and following silencing or stable transfection of the AMP+ gene. **Results:** AMP+ in swine exhibited a high level of homology with HM74A in humans and PUMA-G in mice. BHBA inhibited the LPS-induced secretion of the pro-inflammatory cytokines TNF- α , IL-6 and IL-1 β and the inflammatory protein COX-2 in monocytes of swine. BHBA suppressed the Forskolin-induced cAMP level increase in ST cells, but failed to inhibit the accumulation of cAMP after the AMP+ gene was silenced with shRNA by transfecting cells with the pGPU6-GFP-Neo-AMP+-sus-392 plasmid. BHBA had no effect on cAMP levels in IPEC-J2 cells, but significantly inhibited the increase in cAMP induced by Forskolin treatment following transfection of the AMP+ gene into IPEC-J2 cells by a lentivirus vector. **Conclusion:** Our results indicated that AMP+ encodes a G protein-coupled receptor in *Sus scrofa* that inhibits cAMP levels and mediates anti-inflammatory effects in swine monocytes.

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Introduction

GPR81, GPR109A and GPR109B form a subfamily of G-protein-coupled receptors (GPCRs) for nicotinic acid (niacin) [1-3], and the endogenous ligands of the members of this GPCR subfamily were identified following reports from three research groups demonstrating that GPR109A binds to niacin [4-6]. The ketone body 3-hydroxy-butyrate (BHBA) is reported to be the endogenous ligand of GPR109A [7] and lactate is the endogenous ligand of GPR81 [8, 9]; GPR109B is activated by 3-hydroxylated beta-oxidation intermediates, particularly 3-hydroxy-octanoate [3]. Among the members of this GPCR subfamily, GPR109A has attracted much attention and has been the subject of a large number of studies. GPR109A is a Gi protein-coupled receptor found in humans, rats, mice, hamsters and guinea pigs, whereas there are almost no reports of this protein in other species [4-6, 10]. BHBA is derived from the reduction of acetoacetate in the liver in all species and from the oxidation of butyrate exclusively in the ruminal epithelium. Previous studies have indicated that certain concentrations of BHBA inhibit immune cell-induced inflammation and atherosclerosis by activating of GPR109A; however, high concentrations of BHBA induce cattle hepatocyte inflammatory injury through the NF- κ B signaling pathway, which may be activated by oxidative stress [11]. GPR109A is expressed by adipocytes, monocytes, macrophages, dermal dendritic cells and vascular endothelial cells, but not lymphocytes [4-6, 12-16], and is also present in retinal pigmented and colonic epithelial cells, keratinocytes, microglia, and normal mammary tissue [17-20]. The function of GPR109A was first described in adipocyte lipolysis whereby GPR109A inhibits the activity of adenylate cyclase and subsequently decreases cAMP levels, resulting in reduced protein kinase A and hormone-sensitive lipase activity [5, 21, 22].

Butyrate, which is produced by bowel microbial fermentation of dietary carbohydrates, fiber, proteins and peptides [23-25], functions as the energy source for epithelial cells [26] and also stimulates the growth of small intestinal epithelium [23]. Previous studies have demonstrated that butyrate or sodium butyrate decreases intestinal permeability and enhances the intestinal barrier [27-29]. Intestinal permeability is related to diarrhea, and Fang et al. and Huang et al. observed that sodium butyrate decreases the incidence of diarrhea in weaned piglets, though the mechanism remains unclear [30, 31].

First reported by Uenishi [32], the AMP010014A09 gene is expressed in the porcine thymus, spleen, uterus, lung, liver, ovary and peripheral blood mononuclear cells. As the AMP+ gene shows high homology with the GPR109A gene, we hypothesized that AMP+ encodes an analog of GPR109A in swine that is activated by butyrate, niacin and BHBA. Identification of the butyrate receptor in swine not only will assist efforts to elucidate the mechanism by which butyrate ameliorates intestinal inflammation, the results may provide a potential target for treating piglet diarrhea. Moreover, the findings may also serve as a model for studying the functions and applications of GPR109A.

Materials and Methods

Materials

BHBA (β -Hydroxybutyric acid), LPS (lipopolysaccharide), isobutyl-1-methylxanthine (IBMX) and 4-(3-butoxy-4-methoxy-benzyl) imidazolidone were purchased from Sigma (St. Louis, MO). Forskolin was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). RPMI Medium 1640 and DMEM-F12 (Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient Mixture) were obtained from Gibco (Grand Island, NY, USA). FBS (fetal bovine serum) was purchased from Clark (Australia). Trizol was obtained from Invitrogen (Carlsbad, CA, USA). Ficoll-Paque PLUS was purchased from GE Healthcare (Piscataway, NJ, USA). A PrimeScript RT reagent kit with gDNA Eraser was purchased from Takara (Kyoto, Japan). 2X Taq Master Mix was purchased from Vazyme (Nanjing, China). SYBR Green QuantiTect RT-PCR Kit and X-tremeGENE HP DNA Transfection Reagent were obtained from Roche (South San Francisco, CA, USA). Polybrene was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The lentivirus and the pGPU6-GFP-Neo-AMP+-sus-392 plasmid, which were used to specifically increase AMP+ expression in IPEC-J2 cells and decrease

AMP+ expression in ST cells, respectively, were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All other reagents were of analytical grade or of the highest purity available.

Animals

Landraces used for the experiments were provided by the original breeding pig farm of Jilin University. All experimental animals were approved by the Jilin University Institutional Animal Care and Use Committee, and the experimental procedures were strictly performed according to the Guidelines for the Care and Use of Laboratory Animals in China. All pigs were provided with food and water ad libitum. Six to eight-week-old pigs were used for isolation of monocytes.

Cell culture

Monocytes were isolated from peripheral blood of landraces. Briefly, peripheral blood samples from swine were collected into heparinized collection tubes, diluted 1:2 with Hanks' balanced salt solution, overlaid onto Ficoll-Paque PLUS medium and centrifuged at 400×g for 35 min at 20°C. Next, peripheral blood mononuclear cells (PBMCs) were collected into a sterile centrifuge tube and washed twice with sterile Hanks' solution before centrifuging at 80×g for 10 min at 20°C. The supernatant was removed, and the PBMCs were re-suspended in RPMI 1640 Medium supplemented with 10% (v/v) FBS and maintained at 37°C with 5% CO₂. After four hours, the PBMCs were washed twice using sterile Hanks' solution to remove non-adherent cells. The remaining cells were the monocytes used in experiments.

IPEC-J2, an intestinal epithelial cell line, was a kind gift from Dr. Guohua Wu (College of Animal Science and Technology, China Agricultural University). The swine testicular cell line (ST) isolated from swine fetal testes has been widely used in biomedical research related to pig virus infection. ST cells were purchased from ATCC. IPEC-J2 and ST cells were cultured in complete DMEM/F12 medium with 10% (v/v) FBS. The cells were cultured in an incubator at 37°C in a humidified atmosphere of 5% CO₂.

Treatment of monocytes

LPS (Sigma-Aldrich, USA) was dissolved in phosphate-buffered saline (PBS) and used to induce monocyte secretion of pro-inflammatory cytokines and inflammatory protein. Monocytes were divided into the following four groups: the no treatment (NT) group, the BHBA (1.5 mM) treatment group, the LPS (1 µg/ml) treatment group, and the LPS (1 µg/ml) + BHBA (1.5 mM) treatment group. The cells were cultured in serum-free RPMI 1640 for 3 h to reduce mitogenic effects and then pretreated with BHBA for 1 h before adding LPS. The monocytes were collected at 6 h and 12 h after stimulation to measure expression of the pro-inflammatory cytokines TNF-α, IL-1-β and IL-6 and the inflammatory protein COX-2. Experiments for each of the different treatments were carried out in triplicate.

Real-time quantitative PCR analysis

Total RNA was extracted from cells using Trizol according to the supplier's protocol, and cDNA was synthesized using 2 µg of total RNA. Briefly, 2 µl of Oligo (dT)18 Primer and 2 µg of total RNA were added to the reaction system, which adjusted to a volume of 14 µl using diethyl pyrocarbonate (DEPC) water and mixed at 70°C for 5 min. Next, 4 µl 5X Reverse Transcriptase M-MLV Buffer, 0.5 µl Reverse Transcriptase M-MLV, 1 µl dNTP mixture, and 0.5 µl recombinant RNase inhibitor were added to the reaction system and incubated at 42°C for 90 min and then at 90 °C for 5 min. For PCR amplification, we diluted the cDNA 10 fold and then added 12.5 µl 2x Taq Master Mix, 10.5 µl cDNA, 1 µl sense primer and 1 µl antisense primer. Real-time quantitative RT-PCR (qRT-PCR) was performed using a SYBR Green QuantiTect RT-PCR Kit with specific primers. The reaction was as follows: initial heating step at 94°C for 3 min followed by 40 cycles of two-step reactions at 94°C for 30 s and 60°C for 30 s. The sequences of the primers used in this investigation are shown in Table 1.

Table 1. The primer sequences of TNF-α, IL-1β, IL-6, COX-2, AMP+, and GAPDH

Gene	Sequences	Length (bp)
TNF-α	5'-GCCTCTTCTCCTTCCTCCTG-3'	193
	5'-TCGGCTTTGACATTGGCTAC-3'	
IL-1β	5'-ACCCAAAACCTGGACCTTG-3'	132
	5'-GGGATTCTTCATCGGCTTCT-3'	
IL-6	5'-CTGGGTTCATCAGGAGACC-3'	193
	5'-TTAGGGGTGGTGGCTTTGT-3'	
COX-2	5'-CTCACACTCCTGAACACCTC-3	120
	5'-TAAGCACATCGCACACTCTA-3	
AMP+	5'-GTATGCTGGACCCTTTGGTG-3'	119
	5'-GCTGCGGTTATTGTTGGACT-3'	
GAPDH	5'-GCCATCACTGCCACCCAGAA-3'	153
	5'-GCCAGTGAGCTTCCCGTTGA-3'	

RNA interference and stable transfection

The plasmid pGPU6/GFP/Neo was used as the vector for transfecting AMP+, and the target site of AMP+ was AMP+-sus-392, with a target sequence of 5'-GCCGAATAATGCTCTTCATGT-3'. The sequence of the transcription product was 5'-GCCGAATAATGCTCTTCATGTTTCAAGAGAACATGAAGAGCATTATTTCGGCTT-3'. Thus, the sequence of the recombinant plasmid was predicted. The pGPU6/GFP/Neo vector was used in the NC group. In this case, the target sequence was 5'-GTTCTCCGAACGTGTCACGT-3', and the sequence of the transcription product was 5'-GTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTTCGGAGAATT-3'. Again, the sequence of the recombinant plasmid was as predicted. pGPU6-Neo containing the shRNA oligonucleotides were transfected into ST cells using X-tremeGENE HP DNA Transfection Reagent. The shRNA oligonucleotide most effectively silencing the AMP+ gene was chosen by assessing the degree to which AMP+ mRNA expression was decreased.

A stable transfection process, similar to described in a previous report [33], was used. Briefly, IPEC-J2 cells were seeded into a culture flask and incubated overnight. Next, 20 µl of 1×10^8 TU/ml lentivirus and 8 µl of polybrene were immediately added to the medium. The culture medium was replaced with complete medium after 24 h, and the cells were observed 72-96 h later. The results of stable transfection were authenticated by observing cell fluorescence of cells (the lentivirus expresses green fluorescent protein) and AMP+ expression in the IPEC-J2 cell line.

Immunofluorescence analyses

A total of 5×10^4 cells were transferred onto glass slides pre-coated with rat tail tendon collagen type I and incubated overnight at 37°C with 5% CO₂. Next, we removed the complete culture medium, washed the slides 3 times with cold PBS, added 1 ml immunol staining fix solution and incubated the slides at room temperature for 10 min. The immunol staining fix solution was removed, and the slides were washed 3 times with cold PBS; 50 µl PBS containing 5% (v/v) donkey serum was added, and the slides were incubated for 3 h at room temperature. We diluted the anti-CD14 antibody with PBS containing 5% (v/v) donkey serum, and added 50 µl of the diluted antibody to the glass slides, which were incubated overnight at 4°C. Next, the diluted primary antibody was removed by washing the slides 3 times with cold PBS, after which the fluorescein isothiocyanate (FITC)-conjugated secondary antibody diluted with PBS containing 5% (v/v) donkey serum was applied, and the slides were incubated in the dark for 1 h at room temperature. The secondary antibody was removed by washing the slides 3 times with cold PBS, followed by application of 50 µl DAPI and incubation at room temperature for 15 min. DAPI was removed by washing the slides 3 times with cold PBS, and 20 µl of antifade mounting medium was added; the slide then covered carefully with coverslips. Immunofluorescence microscopy analyses were performed using the requisite, dedicated software

Intracellular cAMP of measurement

A total of 2×10^5 cells/well were seeded into 96-well plates and incubated at 37°C with 5% CO₂ for 12 h. The complete medium was replaced with serum-free medium, and the cells were incubated at 37°C with 5% CO₂ for 3 to reduce mitogenic effects. Forskolin or BHBA was added to the induction buffer, which contained 1×PBS with 500 µM IBMX and 100 µM 4-(3-butoxy-4-methoxy-benzyl) imidazolidone; IBMX and 4-(3-butoxy-4-methoxy-benzyl) imidazolidone are broad-range phosphodiesterase inhibitors that inhibit cAMP hydrolysis. The cells were pretreated or not with forskolin 10 min before BHBA for 10 min. After each of the treatments, cAMP levels were measured using the cAMP-Glo™ assay according to the supplier's protocol. Experiments for each of the different treatments were carried out in triplicate.

Statistics

The results are expressed as means ± SE. Data were analyzed using the statistical software package SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Groups were compared by one-way analysis of variance (ANOVA) followed by the least significant difference test. **P*<0.05 was considered significant, and ***P*<0.01 was considered markedly significant.

Results

AMP+ has a high similarity with HM74A and PUMA-G

To identify AMP+ as an analog of GPR109A, we compared the nucleotide and amino acid sequences of AMP+ with those of HM74A and PUMA-G. AMP+ exhibits nucleotide homology, amino acid identity and amino acid similarity of 86.239%, 82.044% and 90.61% with HM74A. Furthermore, nucleotide homology, amino acid identity, and amino acid similarity between AMP+ and PUMA-G are 82.15%, 82.073% and 87.39% (Fig. 1A and B). The structures of the three proteins were predicted using “SWISS-MODEL” (Fig. 1C-E), and the models showed

Fig. 1. AMP+ has high similarity with HM74A and PUMA-G. (A) The amounts of nucleotides and amino acids in AMP+, HM74A and PUMA-G. (B) Results of the comparisons between nucleotide and amino acid of AMP+, HM74A and PUMA-G. (C) Protein models of AMP+, HM74A and PUMA-G.

Fig. 2. BHBA inhibited LPS-induced increases in levels of pro-inflammatory cytokines and an inflammatory protein in monocytes. (A) Monocytes were identified using an anti-CD14 antibody (green) and DAPI staining (blue). Monocytes were cultured in serum-free RPMI 1640 medium for 3 h to reduce mitogenic effects, pretreated with or without BHBA (1.5 mM) for 1 h, and then treated with or without LPS (1 µg/ml) for 6 and 12 h. (B) Expression of COX-2, LPS treated for 4 h (n=3, *P<0.05, **P<0.01). (C), (D) and (E) Expression of TNF, IL-1β and IL-6 respectively, LPS treated for 6 h (n=3, *P<0.05, **P<0.01). (F), (G) and (H) Expression of TNF, IL-1β and IL-6 respectively, LPS treated for 12 h (n=3, *P<0.05, **P<0.01).

Fig. 3. Effect of BHBA on intracellular cAMP levels in ST cells. (A) Intracellular cAMP levels in control ST cells treated with or without forskolin and BHBA ($n=3$, $*P<0.05$, $**P<0.01$). (B) Transfection results for NC and shRNA plasmid ($n=3$). The shRNA plasmid reduced AMP+ mRNA expression levels, and the NC plasmid failed to decrease AMP+ mRNA expression (C). (D, E) Intracellular cAMP levels in ST cells transfected with the NC and shRNA plasmids ($n=3$, $*P<0.05$, $**P<0.01$).

very minor differences. All data indicated that AMP+ has high homology with HM74A and PUMA-G, despite originating from different species.

BHBA inhibited LPS-induced expression of pro-inflammatory cytokines and an inflammatory protein in monocytes

As demonstrated by immunofluorescence and DAPI staining, most of the cells isolated from swine peripheral blood were monocytes (Fig. 2A). LPS was utilized to induce inflammation in these cells, and BHBA significantly decreased the LPS-induced increase in levels of COX-2 expression (Fig. 2B). In cells pretreated with BHBA for 1 h and then treated with LPS for 6 h, the expression levels of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 were significantly decreased compared to those in the LPS group (Fig. 2C, D and E). Similar inhibition was observed with LPS treatment for 12 h after pretreating with BHBA for 1 h. (Fig. 2F, G and H).

Effects of BHBA on intracellular cAMP levels in ST cells

GPR109A belongs to the Gi family of GPCRs and inhibits the activity of adenylate cyclase following activation by BHBA, which subsequently decreases cAMP levels. Therefore, we utilized BHBA to examine reductions in cAMP levels of ST cells silenced for AMP+. The results indicated that forskolin significantly increased cAMP synthesis and that BHBA significantly attenuated this forskolin-induced cAMP synthesis in ST cells (Fig. 3A). ST cells were transfected with pGPU6-GFP-Neo-AMP+-sus-392 and pGPU6-GFP-Neo-AMP+-sus-NC plasmids (Fig. 3B, C). In the control ST cells transfected with the NC plasmid, no significant change in cAMP levels was observed (Fig. 3D). BHBA failed to inhibit the synthesis of cAMP in ST cells transfected with plasmid containing AMP+ shRNA. (Fig. 3E).

Stable transfection of the AMP+ gene into IPEC-J2 cell

We had previously confirmed that several tissues of swine express AMP+, but that AMP+ was not expressed in the IPEC-J2 (NT) (Fig. 4A), we presumed that AMP+ was not

Fig. 4. Stable transfection of the AMP+ gene into IPEC-J2 cells. (A) PCR results for IPEC-J2 (NT), NC-IPEC-J2 (NC) and AMP+-IPEC-J2 (AMP+) cells. (B) Lentiviral transfection results for NC and AMP+. Green indicates positively transfected cells.

Fig. 5. Effects of BHBA on intracellular cAMP levels in IPEC-J2 cells. (A) Intracellular cAMP levels of IPEC-J2 cells treated with or without forskolin and BHBA (n=3, * P <0.05, ** P <0.01). (B) Intracellular cAMP levels of NC-IPEC-J2 cells treated with or without forskolin and BHBA (n=3, * P <0.05, ** P <0.01). (C) Intracellular cAMP levels of AMP+-IPEC-J2 cells treated with or without forskolin and BHBA (n=3, * P <0.05, ** P <0.01).

expressed in the IPEC-J2 cell line, we transfected AMP+ into IPEC-J2 cells using a lentivirus vector containing the gene. According to our results, IPEC-J2 (NT) and NC-IPEC-J2 cells did not express AMP+, whereas AMP+-IPEC-J2 cells did express AMP+ (Fig. 4A). The lentivirus produces green fluorescence protein, which facilitates observation of positively transfected cells by fluorescence microscopy (Fig. 4B). Microscopy and PCR indicated that we had successfully transfected AMP+ into IPEC-J2 cells and the transfection efficiency was very high.

cAMP levels were inhibited by BHBA in IPEC-J2 cells transfected with AMP+.

We treated IPEC-J2, NC-IPEC-J2 and AMP+-IPEC-J2 cells with BHBA and forskolin. Compared with the NT group, BHBA had no effect on cAMP levels in the control-IPEC-J2 cells, whereas forskolin increased intracellular cAMP levels (Fig. 5A). The same results were observed in NC-IPEC-J2 cells (Fig. 5B): BHBA significantly decreased cAMP levels in AMP+-IPEC-J2 cells with or without forskolin treatment (Fig. 5C).

Discussion

LPS has been widely used to establish *in vitro* models of inflammation model [34, 35]. In this study, LPS was used to induce secretion of pro-inflammatory cytokines and an

inflammatory protein in monocytes. The results indicated that BHBA inhibited LPS-induced release of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 and the inflammatory protein COX-2. This is consistent with our previous results that BHBA suppresses the LPS-induced release of TNF- α , IL-1 β and IL-6 via GPR109A in primary rat microglial cells and BV-2 cells [36, 37]. Similarly, niacin has been demonstrated to exert potent anti-inflammatory effects in human adipocytes and monocytes through GPR109A-dependent mechanisms [38, 39]. In addition, niacin and BHBA suppress the TNF- α -induced release of pro-inflammatory cytokines by GPR109A in retinal pigment epithelial cells [18]. These studies highlight the role of GPR109A in anti-inflammation. Therefore, we hypothesized that GPR109A or its' analog is also present in swine monocytes.

Niacin rapidly reduced cAMP levels in human basal macrophages pre-incubated with or without pertussis toxin (PTX) for 18 h; although cAMP levels were reduced in the niacin group, the effect was not observed in the PTX group [40]. Niacin also inhibited intracellular cAMP synthesis induced by forskolin in mouse primary hepatocytes [41]. These reports indicate that activation of GPR109A decreases cAMP levels, this consistent with our results that BHBA reduced the forskolin-induced increases in intracellular cAMP levels in ST cells. Our results also indicated that ST cells contain a receptor or analog that can be activated by BHBA, resulting in a subsequent decrease in intracellular cAMP levels. Moreover, silencing of the AMP⁺ gene with shRNA abolished the inhibitory effect of BHBA on cAMP levels. Next, we observed that BHBA had no effects on the cAMP levels in IPEC-J2 cells, which do not express the AMP⁺ gene. However, when we transfected the AMP⁺ gene into these cells, BHBA decreased forskolin-induced increases in intracellular cAMP levels. Our results are similar to previous reports demonstrating that GPR109A is activated upon binding of niacin and functions in a G protein-coupled manner to decrease cAMP production in GPR109A-expressing Chinese hamster ovary-K1 cells [5]. Niacin also inhibited forskolin-induced cAMP synthesis in GPR109A-expressing HEK-293 cells, but this suppression was abolished when the cells were pretreated with PTX [42, 43]. Taken together, the results from previous studies and the current study led us to hypothesize that AMP⁺ is activated by BHBA, causing decreases in intracellular cAMP levels. The results also indicated that AMP⁺ is the GPR109A analog in swine and can be activated by BHBA.

Our findings not only reveal the potential mechanism of by which sodium butyrate ameliorates diarrhea in weaned piglets but also provide a powerful evidence for the presence of GPR109A in various species. Previous studies have indicated that niacin reduces inflammation in atherosclerosis [44], sepsis [45], obesity [46], diabetic retinopathy [18] and renal disease [47] via activation of GPR109A. Therefore, our study provides a model for investigating the functions and applications of GPR109A. For example, in vascular disease, niacin has been used as an antidyslipidemic drug to prevent and treat atherosclerosis, effects that are mediated by immune cell GPR109A [44, 48, 49]. Our findings in swine will may provide the basis for a more appropriate animal model for atherosclerosis than mouse models because swine is a larger mammal, compared to mice, has more physiological similarities to humans.

Conclusion

The results of our experiments provide strong evidence indicating that the AMP⁺ gene encodes a GPCR in swine that has high homology with GPR109A.

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Disclosure Statement

The authors declare no conflict of interest.

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