Research Paper

Glucagon-producing cells are increased in Mas-deficient mice.

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SUMMARY:

It has been shown that Angiotensin-(1-7) [Ang-(1-7)] produces several effects related to glucose homeostasis. In this study, we aimed to investigate the effects of genetic deletion of Ang-(1-7), the GPCR Mas, on the glucagon – producing cells. C57BL6/N Mas$^{-/-}$ mice presented a significant and marked increase in pancreatic α-cells (number of cells: 146±21 vs. 67±8 in WT - p<0.001) and the percentage per islet (17.9±0.91 vs. 12.3±0.9% in WT - p<0.0001) with subsequent reduction of β-cells percentage (82.1±0.91 vs. 87.7±0.9% in WT - p<0.0001). Accordingly, glucagon plasma levels were increased (516.7 ± 36.35 vs 390.8 ± 56.45 pg/mL in WT, p<0.05) and insulin plasma levels were decreased in C57BL6/N Mas$^{-/-}$ mice (0.25 ± 0.01 vs 0.31 ± 56.45 pg/mL in WT- p=0.02). In order to eliminate the possibility of a background-related phenotype we determined the number of glucagon-producing cells in FVB/N Mas$^{-/-}$ mice. In keeping with the observations in C57BL6/N Mas$^{-/-}$ mice, the number and percentage of pancreatic α-cells were also significantly increased in these mice (number of α-cells: 260±22 vs. 156±12 in WT, p<0.001; percentage per islet: 16±0.8 vs. 10±0.5% in WT, p<0.0001). These results suggest that Mas has a previously unexpected role on the pancreatic glucagon production.

INTRODUCTION:

Besides its role in the regulation of blood pressure and the cardiovascular system, the renin-angiotensin system (RAS) plays an important role in the control of glucose and lipid metabolism (1). Moreover, it is determinant in the pathophysiology of diabetes and metabolic syndrome (2).

In the past few years, our understanding of the role of the heptapeptide Angiotensin-(1-7) has been extended to include other organs and systems well beyond the heart, vessels and the kidney (3). A relevant part of this new understanding is derived from the phenotypic characterization of Mas-deficient mice in a variety of models, tissues and conditions, including the cardiovascular system (4), liver (5), testis (6), memory process (7), erectile dysfunction (8) and inflammation (9,10).

Angiotensin- (1-7) has been described to improve the glucose homeostasis by influencing different mechanisms including: improvement of insulin signaling in skeletal muscle (11), endothelium (12), adipose tissue and liver (13). Moreover, *Mas* deletion has been reported to have deleterious effects on glucose and lipid metabolism (14). Although it is now clear, in contrast to ACE/Ang II/AT1R, the ACE2/Ang-(1-7)/Mas axis has a beneficial action on glucose metabolism (15, 16, 17, 18), the mechanism of the effect of Ang-(1-7)/Mas action regarding insulin-related mechanisms is not fully understood.

In this study we aimed to further address this issue by determining the effect of Mas deletion on the α and β cell population in the pancreas. In order to rule out background dependency the study was performed in two mouse lines: C57BL6/N and FVB/N Mas-deficient (Mas-KO) mice.

RESEARCH DESIGN AND METHODS:

Animals

12 weeks-old male Mas-KO (both FVB/N and BL6/N, n=10) and Wild-Type (WT, n=8) mice, weighting approximately 25g, were obtained from the Biological Sciences Institute of Federal University of Minas Gerais (ICB-UFMG, Brazil). Mice were kept under standard and controlled environment conditions of temperature (20ºC - 25ºC), light and dark cycles (12 hours each, 06:00 - 18:00) with standard chow and water ad libitum. The experimental protocol was approved by the ethic committee in animal experimentation of Federal University of Minas Gerais (147/2008).

Pancreas dissection and Immunohistochemistry

After overnight fasting, mice were sacrificed by i.p. administration of a ketamine (130 mg/kg) and xylazine (0.3 mg/kg) solution. The pancreas was dissected and the splenic part was fixed in 10% formalin for 48hs, dehydrated in ethanol, and embedded in paraffin. Tissues were cut with 5 µm thick, with 150 µm interval between the cuts to obtain different pancreatic islets, and mounted on glass slides previously prepared to prevent detachment of cuts during the immunostaining. The slices were deparaffinized in xylene, rehydrated in alcohol baths decreasing concentration and washed in deionized water. Subsequently, the slices were processed for retrieving epitopes using the microwave oven in citric acid solution, pH 6.0 for 15 minutes. Next, the sections were treated with 3% hydrogen peroxide (H_2O_2) for 30 minutes to inhibit endogenous peroxidase. The tissues were incubated with anti-glucagon primary antibody (1:200 – Abcam) at 4ºC overnight. The next day, the sections were incubated with the secondary antibody biotinylated (1:200 – Vector Laboratories) for 1 hour, and the slices were incubated with reagents of the ABC detection system, as specified by the manufacturer. Peroxidase activity was visualized by exposing the sections to DAB solution [(3-3)-diaminobenzidine tetrahydrochloride (Sigma)]. Thereafter, the sections were counterstained with Mayer hematoxylin

dehydrated in ethanol and mounted. In the negative control, the primary antibody was replaced by nonimmune serum.

Glucagon Levels and Insulin:

Enzyme-linked immunosorbent assay (ELISA) kit was used to measure serum insulin (Linco Research, St. Charles) and plasma glucagon (ALPCO immunoassays) levels.

Image analysis:

Positive immunostaining for pancreatic α-cell was examined using a light microscope (Zeiss). Ten islets per mouse pancreas were randomly chosen and all the cells present in the islet were counted. After that, each marked α-cell was also counted. The estimated β-cell content was obtained subtracting the total cells from the α-cells.

Statistical Analysis

Data were analysed using non-paired Student t test in the GraphPad Prism (GraphPad Software; San Diego, CA, USA). Data are expressed as mean ± SEM and Statistical significance was accepted at the level of $p<0.05$.

RESULTS

As shown in Figure 1A, Mas-deficiency in the FVB/N background resulted in an increase in the total number and percentage of the pancreatic α-cell when compared to wild-type mice (number of α-cells: 260±22 vs. 156±12 in WT, $p<0.001$; percentage per islet: 16±0.8 vs. 10±0.5% in WT, $p<0.0001$). Likewise the number and percentage of α-cells were significantly higher in the C57BL6/N in comparison to the WT mice (Figure 1B). The number of α-cells in C57BL6/N mice averaged is 146±21 vs. 67±8 in WT ($p<0.001$) and the percentage per islet is 17.9±0.91 vs. 12.3±0.9% in WT ($p<0.0001$). Regarding the estimated β-cell number, no significantly differences was evidenced in FVB/N and C57BL6/N mice, however, the percentage of β-cells was reduced in both backgrounds (C57BL6/N: 82.1±0.91 vs. 87.7±0.9% in WT $p<0.0001$; FVB/N: 83.53±1.2 vs.

88.6±0.9% in WT, p<0.001, Figure 2). No significant differences were observed in the total amount of cells in pancreatic islets, although a trend for increase in Mas-KO was observed (Figure 3). These results indicate that the increment in the total cell number, independently of the strain background, is linked to increase in the α-pancreatic cells. In keeping with the morphological data, glucagon plasma level was increased in C57BL6/N background: 516.7 ± 36.35 vs 390.8 ± 56.45 in pg/mL vs WT 0.25 ± 0.01 vs 0.31 ± 56.45 pg/mL (p<0.05) while insulin 0.25 ± 0.01 vs 0.31 ± 56.45 pg/mL (p=0.02) in WT was reduced (Figure 4).

DISCUSSION

In this study, we report for the first time that genetic ablation of Mas produced a marked alteration in the total number and percentage of glucagon-producing cell in the pancreas. This was associated to a significant increase of fasting plasma glucagon levels. As consequence of the increase of α-cells the percentage of β-cells was reduced in the pancreas since the absolute number did not differ from that of WT mice. In the past few years, the role of Ang-(1-7)/Mas axis in the attenuation of metabolic diseases has been investigated in different models of diabetes and also in different experimental contexts (19,20). Wang and colleagues shed light on the pivotal role of ACE2/Ang-(1–7)/Mas axis in organogenesis during embryonic development of pancreatic islets. In this study, the authors also demonstrated a major role of Mas receptor in the pancreatic development and insulin secretion in early post-natal life reiterating the potential implications of the Ang-(1-7)/Mas axis for the development of therapies to handle pathological metabolic states (21).

In keeping with of our current observation, we have previously described that Mas-KO mice presented significant changes in blood glucose homeostasis (14). In this previous study, however, we did not address the possibility of changes in glucagon as one of the mechanisms of such alterations. Although, functional studies involving glucagon-related mechanisms are obviously needed to fully understand its role in the metabolic changes observed in Mas-KO mice, our current data indicate that an imbalance of α and β cells is involved in the functional phenotypic changes of Mas-KO mice.

In previous studies, significant differences were reported for the phenotypic variation in the two backgrounds where Mas has been deleted: FVB/N and C57BL6/N. For instance, increase in blood pressure was observed in FVB/N but not in C57BL6/N mice (22), and the metabolic syndrome-like state observed in FVB/N mice was absent in the BL6/N background (14). Here we show that the increase in glucagon-producing cells is a common feature of both backgrounds, clearly indicating that this phenotypic alteration is mostly linked to Mas deletion. A further implication of this finding is that Mas appears to play a key role in the glucagon-producing pancreatic cells.

Diabetes mellitus is characterized by insulin insufficiency due to either decreased insulin release or end-organ insulin resistance. Among the major existing categories of diabetes, type I (T1DM) usually occurs in children and young adults and is characterized by pancreatic β-cell failure or autoimmune destruction of the pancreatic β-cells (18). Indeed, it has been shown that glucagon antagonism, glucagon neutralizing antibodies and antisense oligonucleotides lower plasma glucose level in many rodent models of diabetes (23,24,25,26). Similarly, the reversal of diabetes induced by leptin therapy in rodents has been attributed to a reduction in plasma glucagon (27,28,29). In keeping with studies in rodents, suppression of glucagon in patients with diabetes improves glucose tolerance (30,31). These observations support a role for glucagon, and probably Mas, in diabetes hyperglycemia.

CONCLUSION:

The main finding of this report is the marked increase in the number and percent of pancreatic α-cells in Mas-deficient mice. This was associated to a significant increase in glucagon plasma levels. These findings suggest a crucial role for Mas in the pancreatic morphology with subsequent impact in glucose homeostasis. Although further studies are necessary to fully understand the role of Ang-(1-7)/Mas in the endocrine pancreas, our results provide a clear basis for understanding the role of Mas in glucagon production.

Conflict of interests and competing financial interests
None.

Author contributions
JFB coordinates the study. She participates in all experimental procedures, data collections and analysis and has drafted this manuscript.

DRD participates in the data collection, analysis and has drafted this manuscript.

NA e MB participates in the all experimental procedures, data interpretation and reviewed and approved this manuscript.

RAS is the main investigator of the study. He participates in the coordination of all tasks, designed and led the study, raised funding for the study, participates in data collection and analysis procedures, and has drafted, reviewed and approved the manuscript.

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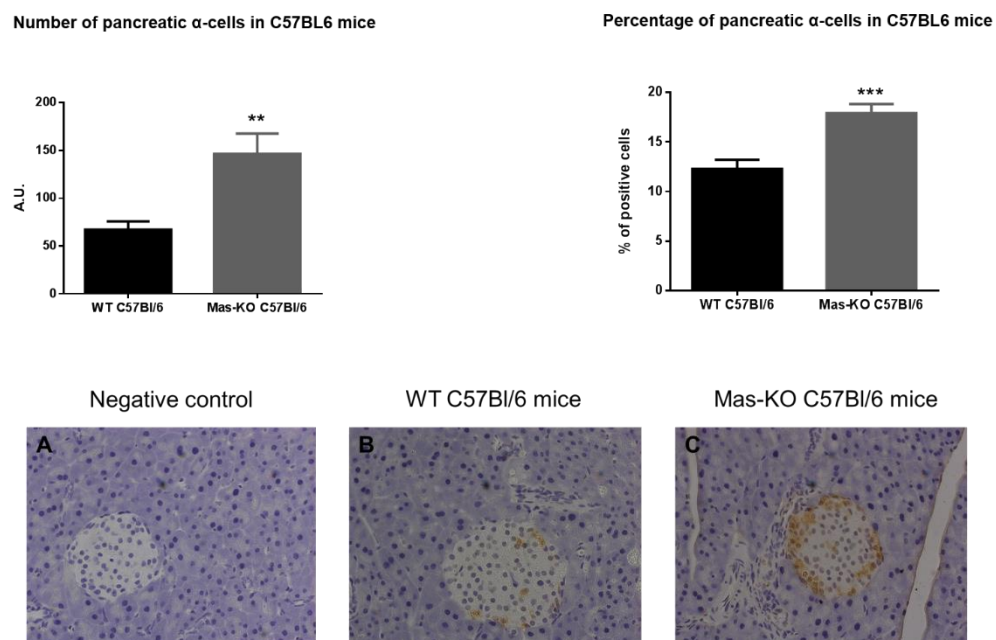
Figure 1A: Total number and percentage of pancreatic α-cells in FVB/N Mas-KO in comparison with WT mice;

1. A
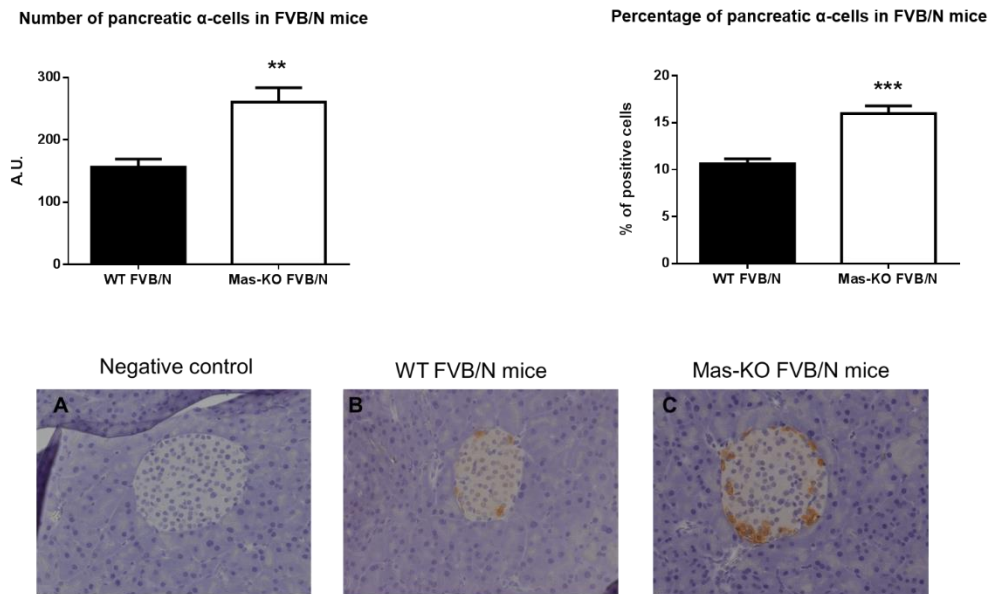
**Figurre 1B: Total number and percentage of α-cells in C57BL6 Mas-KO mice vs. WT**.

1.B

1.A and 1.B: Values are presented as means ± SEM Images represent the immunohistochemical staining of α-cell in mouse pancreatic islet of FVB/N and C57BL6 Mas-KO mice **(A) Negative control**: replacement of primary antibody
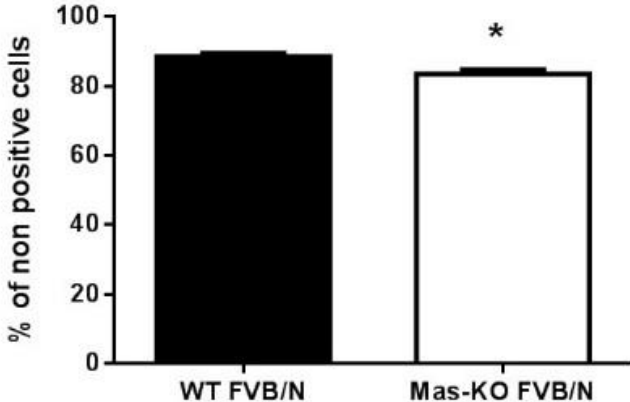
with buffer; **(B)** immuno staining for pancreatic α-cells in **WT**; **(C) Mas-KO** mice, respectively. Magnification: 40X.
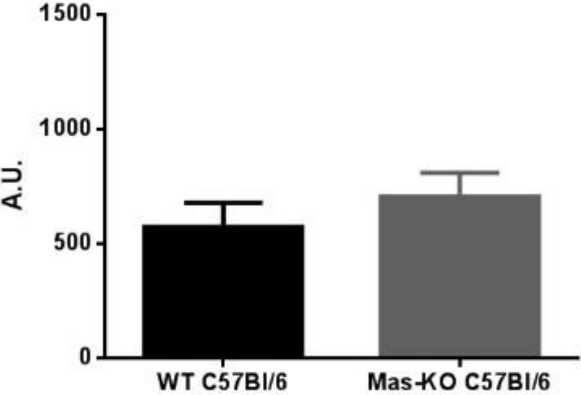
Figure 2:

A

Number of pancreatic β-cells in FVB/N mice
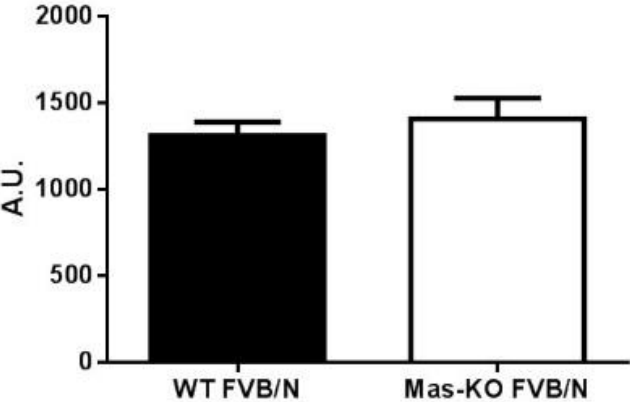
Estimated percentage of pancreatic β-cells in FVB/N mice

B

Number of pancreatic β-cells in C57BL6 mice

Estimated percentage of pancreatic β-cells in C57BL6 mice

Figure 3:

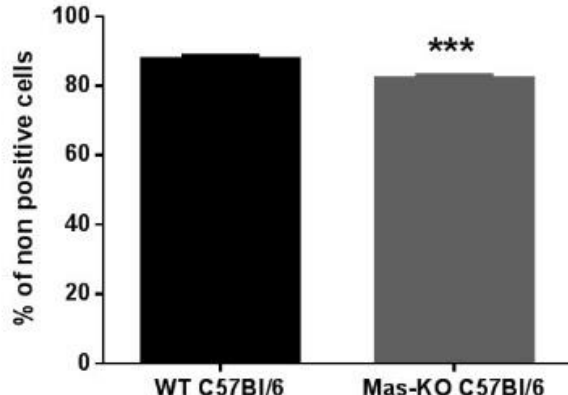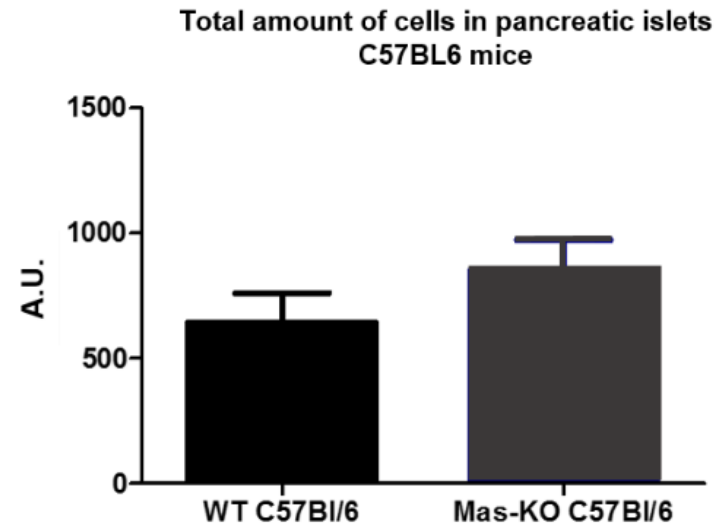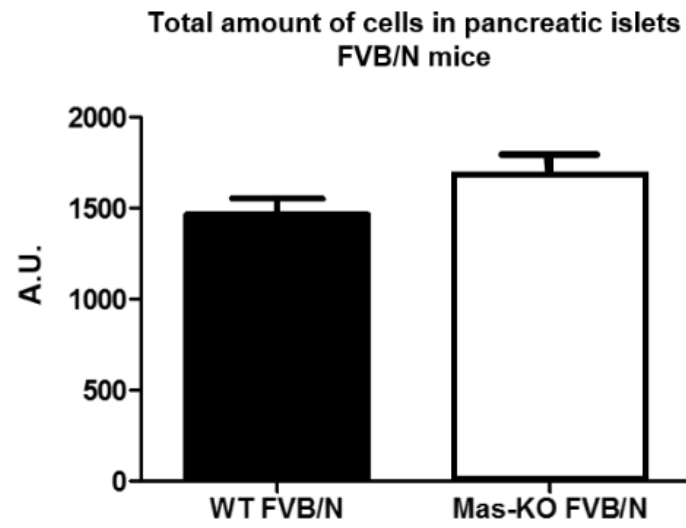Total amount of cells in pancreatic islets FVB/N mice

Total amount of cells in pancreatic islets C57BL6 mice

Figure 4:

Glucagon levels

WT C57Bl/6   Mas-KO C57Bl/6

pg/ml

Insulin levels

WT C57Bl/6   Mas-KO C57Bl/6

pg/ml